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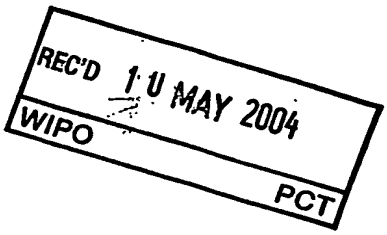

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Therapeutic products with enhanced ability to immunomodulate cell functions

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THERAPEUTIC PRODUCTS WITH ENHANCED ABILITY TO IMMUNOMODULATE CELL FUNCTIONS

The present invention relates to a method for the production and the selection of human or chimæric or humanized antibodies or molecules that comprise the Fc region of human IgG, capable of modulating the activity of one or several particular Fc receptors, such as the triggering of inhibitory functions through the human type II receptors of IgG (FcgammaRII/CD32).

INTRODUCTION

Antibody based therapy in human has emerged with the possibility of producing partially human antibodies (chimeric antibodies) and more recently humanized antibodies. Humanized or murine/human chimeric recombinant antibodies have been developed to prevent the appearance of human anti-mouse antibodies ("HAMA") in patients treated with mouse antibodies, thus avoiding side-effects due to the formation of immune-complexes between HAMA and the injected antibody. In addition, such engineered antibodies exhibit stronger effector functions than their murine counterparts, as their binding to human FcgammaR is improved. Numerous antibodies are currently being tested in clinical trials for the treatment of cancer for example. But, as of today, only few antibodies have shown efficacy at low dose and several trials have been prematurely terminated. Higher dose antibody administration is mainly limited by two factors. First, it increases likewise numerous side effects. Second, it is still a problem to produce large quantities of antibodies and the cost associated with scaling-up is not always economically viable.

These problems need to be addressed before applying antibodies therapy to large population afflicted with various diseases.

It has been shown that the Fc region of IgG is essential for the functions of antibodies. Th recruitment of C1q and of membrane FcgammaR through the Fc region of IgG allows t trigger various effector mechanisms such as cytotoxicity, cytokine release or endocytosi For instance, it has been shown that human IgG1, the most represented human Ig subclasses, that comprise IgG1, IgG2, IgG3 and IgG4, triggers the highest antibody dependent cell cytotoxicity (ADCC). It is due to the ability of human IgG1 to efficientl bind to FcgammaR expressed on NK cells and monocytes/macrophages. In additio FcgammaR play an important role in immune regulation by triggering inhibitory functions. Three distinct classes of FcgammaR have been defined both in humans and mice. Huma FcgammaR include the high affinity FcgammaRI (CD64), and the low-affinity FcgammaR FcgammaRII (CD32) and FcgammaRIII (CD16). Three genes encode FcgammaRI isoform (A, B, C), three genes encode FcgammaRII isoforms (A1, B1, B2) and two genes encod FcgammaRIII isoforms (A, B). Attempts have been made to improve the efficacy of IgG antibodies by modifying the amino-acid sequence in the Fc domain so that a modulation o the interactions of Fc region with FcgammaR can be achieved (see for example WO 99/54572).

Biophysical and molecular studies have indicated that several amino-acid residues located i the hinge region between the CH1 and CH2 domains and immediately adjacent to the N terminus of the CH2 domain of IgG1, as well as the sugar chain linked to the CH2 domain a position Asn^{297}, play a critical role in FcgammaR binding. On the one hand, a common se of IgG1 residues is involved in binding to all FcgammaR (I, II, III), but residues outside thi common set were identified when FcgammaRII and FcgammaRIII interactions with huma Fcgamma1 were studied in details (Shields et al., J. Biol. Chem., 276, 6591-6604, 2001). O the other hand, although not in direct contact with the FcgammaR, the carbohydrate attache to the conserved residue Asn^{297} on Fc is likely to stabilize the conformation of the recepto binding epitope on Fc (Radaev et al., J. Biol. Chem., 276, 16469-16477, 2001). It has bee hypothesized that deglycosylation causes a conformational change in the relative orientatio of the two CH2 domains such as the Fc transitions from an open to a closed conformation preventing FcgammaR binding (Radaev & Sun, J. Biol. Chem., 276, 16478-16483, 2001)

Analysis of IgG1 glycoforms bearing consecutively truncated oligosaccharides confirmed that removal of sugar residues permits the mutual approach of CH2 domains resulting in the generation of a closed conformation (Krapp et al., J. Mol. Biol., 325, 979-989, 2003).

Engineered IgG glycoforms have been shown to trigger optimized ADCC through the recruitment of FcgammaRIII. First, Umana et al. proposed that an IgG1 antibody engineered to contain increasing amounts of bisected complex oligosaccharides (bisecting N-acetylglucosamine, GlcNAC) would allow to trigger a strong ADCC as compared to its parental counterpart (Umana et al., Nature Biotechnol., 17, 176-180, 1999), although this claim has been challenged (Shinkawa et al., J. Biol. Chem, 2002).

Such contradictory results show that it is difficult to identify the actual optimized oligosaccharides structures responsible for the activation or inhibition of a given FcR We found that it is more relevant to identify patterns involved in improved binding rather than focusing on a unique structure. Indeed, we have observed that binding occurs within a range of similar structures.

In this regard, we have demonstrated that particular glycosylation patterns attached to the Fc domain are responsible for enhancing Fc region - FcRIII receptors interaction. In this regard, we filed WO 01/77181 showing that it is possible to prepare compositions of antibodies with improved ADCC properties from particular cell lines, such as YB2/0, and demonstrating the role of particular glycosylation patterns. Such antibodies present short oligosaccharide chains, a weak sialylation, no or weak level of bisecting GlcNac and a low level of fucose between 20% and 50%.

It was also postulated in WO 00/61739 that the presence or the absence of fucose modulates the activity of antibodies. The lack of fucose on human IgG1 N-linked oligosaccharides has been shown to improve FcgammaRIII binding and ADCC. Recombinant human IgG1 produced in YB2/0 cells (Shinkawa et al., J. Biol. Chem., 2002) or in CHO-Lec13 cells (Shields et al., J. Biol. Chem., 277, 26733-26740, 2002), which exhibited a low-fucose

content or were deficient in fucose as compared to the same IgG1 produced in wild-typ CHO cells, showed an enhanced ability to trigger cellular cytotoxicity. By contrast, correlation between galactose and ADCC was not observed and the content of bisectin GlcNAC only marginally affected ADCC (Shinkawa et al., J. Biol. Chem., 2002).

While the above results confirm what we previously described in our application W(01/77181, we now have evidence showing that the alteration of antibody activity can not b resumed solely at the fucose level. The implication of GlcNac, mannose, sialic aci galactose and their respective position is extremely variable in antibodies produced fro different cell lines. This observation confirms that different glycosylation patterns are n only involved in Fc region - Fc receptors interaction but are also responsible for the functio of the antibody.

Therefore, the general purpose of the invention is to provide a method for fine-tuning th function of a given antibody in respect to the different Fc receptors. Different define patterns would allow the modulation of the different FcR.

Among the receptors for the Fc region of IgG, type IIB Fcgamma receptors is of particula interest. FcRII are single-chain, low-affinity receptors that bind with increased affinity IgG present in immune-complexes, or bound to cell surface antigens. Genetic and protei analyses have shown that FcgammaRIIB are present under two isoforms in Human (FcgammaRIIB1 and FcgammaRIIB2), generated by alternative splicing of the three exon that encode the intracytoplasmic domain of the receptor. FcgammaRIIB are expressed on cells, monocytes, dendritic cells, mast cells and basophils. By contrast to all the other know FcgammaR, FcgammaRIIB isoforms inhibit cell activation induced through activatin receptors (Amigorena et al., Science, 256, 1808-1812, 1992). Molecular analyses hav shown that the presence of an YxxL motif, termed "immunoreceptor tyrosine-base inhibition motif" (ITIM) in the intracellular domain of FcgammaRIIB is directly involved i the inhibitory function of the receptor. When tyrosyl phosphorylated, ITIM binds SH domain of phosphatases, namely SHP-1, SHP-2 and the phosphatidylinositol polyphosphat

5-phosphatase, SHIP. SHIP can dephosphorylate PIP3 into PI(3,4)P2, preventing the recruitment of the kinase *btk* which phosphorylates phospholipase C-gamma (PLC gamma). The production of inositol (1,4,5)-tri-phosphate (IP3) is then blocked. It leads to the inhibition of the influx of extracellular calcium, an early event triggered by the recruitment of activating receptors. It also inhibits late events such as cytokine production or cell proliferation (reviewed in O. Malbec et al., in "Immunoreceptor Tyrosine-based Inhibition Motifs", M. Daëron & E. Vivier eds, 1999, Springer-Verlag, Berlin, pp.13-27).

The biological significance of FcgammaRII-dependent negative regulation has been examined by a number of authors. It has been shown that negative regulation of antibody responses can be achieved through the recruitment of FcgammaRIIB, although it is likely that other mechanisms concur in the negative regulation of antibody responses. Thus, one can suggest that antibody-based auto-immune diseases or unwanted allo-immunization leading to the appearance of pathogenic antibodies could be circumvented through an optimized recruitment of FcgammaRIIB by antibodies or other molecules capable of binding to FcgammaRIIB.

In addition, the recruitment of FcgammaRIIB present on mast cells and basophils by allergen-complexed IgG and the subsequent co-aggregation of FcgammaRIIB with FcepsilonRI, once IgE are complexed to the same allergen, is responsible for the blockade of the anaphylactic response by these cells. Thus, IgG antibodies with an optimized ability to engage FcgammaRIIB and, hence, to trigger FcgammaRIIB inhibitory functions could represent efficient therapeutic tools in the treatment of allergy.

Finally, the inhibitory functions triggered by the recruitment of FcgammaRIIB could be exploited in cancer patients. On the one hand, it has been shown that FcgammaRIIB K.O. mice exhibit better anti-tumor responses in models where immuno-deficient FcgammaRIIB$^{-/-}$ mice were tumor-engrafted and treated with recombinant monoclonal antibodies such as anti-CD20 or anti-HER2/Neu chimaeric or humanized antibodies, respectively. Thus, monoclonal IgG antibodies unable to trigger strong FcgammaRII inhibitory functions might

represent optimized tools in tumor treatments. On the other hand, FcgammaRIIb negative regulate hematopoietic cell proliferation dependent on Receptor Tyrosine Kinases (RTKs Bifunctional molecules such as anti-RTK IgG antibodies with optimized Fc region capab of efficiently co-aggregating RTKs with FcgammaRIIB could therefore block th proliferation of FcgammaRIIB$^+$ tumor cells.

The impact of glycosylation on FcgammaRII/human IgG interaction has also bee documented using soluble FcgammaR ectodomains. Both for mouse FcgammaRII an human FcgammaRIIB, a 1:1 stoichiometry has been deduced from sedimentatio equilibrium technique with ultracentrifugation and isothermal titration calorimetry (ITC respectively (Mimura et al., J. Biol. Chem., 276, 45539-45547, 2001; Kato et al., J. Mo Biol., 295, 213-224, 2001). Interactions between Asn^{297} and the primary GlcNAC residu were shown to be important for recognition of human chimeric IgG3 by huma FcgammaRII (Lund et al., FASEB J., 116, 115-119, 1995). Replacement of contact residue for galactose on the alpha(1-6) mannose arm does not affect FcgammaRII recognition whil replacement of Asp^{265}, a contact for a core GlcNAC residue, results in a loss of F gammaRII recognition (Jefferis et al., Immunol. Letters, 44, 111-117, 1995). In addition another study indicated that GlcNAC residues contribute only slightly to receptor binding o human IgG1, whereas removal of alpha(1-3)- and alpha(1-6)-arm mannose residues result in a significantly decreased affinity (Mimura et al., J. Biol. Chem., 276, 45539-4554 2001). The same study concluded that the truncation of oligosaccharides causes a close disposition of the two CH2 domains and is accompanied by a decreased binding t FcgammaIIB. By contrast to FcgammaRIII, antibodies produced in CHO-Lec13 cells, thu devoided of fucose, showed only a slight improvement in binding to the solubl immobilized Arg^{131}-FcgammaRIIA polymorphic form and to the soluble FcgammaRII form, and none to the soluble His^{131}-FcgammaRIIA polymorphic form (both of ther corresponding to the extracellular and transmembrane domains). Since the former receptor have arginine at position 131, it was postulated that the fucose may interact directly with th FcRgammaII residue at this position or alter the IgG1 conformation so that a slight negativ effect on FcgammaRII binding is induced by its presence (Shields et al., J. Biol. Chem., 27

26733-26740, 2002). The same study suggested that the galactose content does not affect binding to FcgammaRII.

Here, we propose to introduce changes in the glycosylation or to purify adequate glycoforms of human IgG produced for therapeutic use so as to prepare antibodies capable of exerting a fine tuning of FcgammaRIIB immuno-regulatory negative functions for example. Such method allows for the first time to identify suitable cell lines for producing antibodies with enhanced efficacy and specificity. Purification of different antibody glycoforms relies on the use of immobilized soluble forms of FcgammaR that makes it possible to enrich for antibodies with an optimized or a decreased ability to bind and recruit FcgammaR.

DISCLOSURE OF THE INVENTION

The present invention provides a method for producing and selecting human recombinant antibodies or chimaeric antibodies or humanized antibodies or molecules that comprise Fc region of human IgG with defined glycosylation patterns by binding assays - immunofluorescence and ELISA - that use different FcR, in particular FcgammaRIIB as well as FcgammaRIII. For example, this method can be applied for selecting human recombinant antibodies or antibody-derived molecules with defined glycosylation patterns, capable of specifically triggering inhibitory functions through the human type II receptors for the Fc region of IgG (FcgammaRII/CD32) by functional assays such as calcium mobilization assay and cytokine secretion assay.

The invention is based on the production of recombinant human, humanized or chimaeric antibodies with different glycosylation patterns by various cell lines cultured in defined media leading to specialized and fine-tuned antibodies.

In a first aspect, the invention is directed to a method for the preparation of human, humanized or chimæric antibodies or polypeptides comprising Fc region of human IgG with

enhanced therapeutic properties and decreased side effects, wherein said method comprise the step consisting of:

a) providing candidate human, humanized or chimæric antibodies or polypeptide comprising Fc region of human IgG produced from different cell lines selected fro hybridoma, heterohybridoma, animal cell lines or eukaryote microorganisms, transfecte with a vector comprising the coding sequence for said antibody or polypeptide,

b) testing the binding of said antibodies or polypeptides on Fcgamma receptors includin CD16 (FcgammaRIII) and CD32 (FcgammaRIIA and B),

c) selecting antibodies or polypeptides which :

i) bind specifically or preferentially to a first Fcgamma receptor,

ii) bind to both a first Fcgamma receptor and a second Fcgamma receptor,

iii) do not bind or bind only weakly to both a first Fcgamma receptor and a second Fcgamm receptor, or

iv) bind specifically or preferentially to said second Fcgamma receptor.

For example, step c) may consist of selecting antibodies or polypeptides which:

i) bind specifically or preferentially to CD16,

ii) bind to both CD16 and CD32,

iii) do not bind or bind only weakly to both CD16 and CD32, or

iv) bind specifically or preferentially to CD32.

The expression "Fc gamma receptors" refers to all Fc receptors family, including FcRγII FcγRIIB, FcγRIIIA, FcgammaRIIIB and FcγRIA, FcγRIB and FcγRIC.

For example, the binding assays can be performed using FcgammaRIII and FcgammaRIIA or FcgammaRIII, FcgammaRIIA and FcgammaRIIB.

The binding assays can be performed using :
i) indicator cells from cell lines that express different Fc receptors on their cell surface,
ii) recombinant Fc receptors comprising FcgammaR ectodomains, Fc receptors derived-peptides or in an assay reaction mixture comprising a soluble Fc receptors or fragment thereof.
For example, one of the Fc receptors can be FcgammaRIIB and another is FcgammaRIII.

The binding assay can be followed with a functional assay. In this regard, the subset of antibodies or polypeptides selected in step c) for the feature (iv) or (ii), more particularly the subset of antibodies or polypeptides selected to bind specifically or preferentially CD32 (iv) or both CD16 and CD32 (ii), are further tested and selected for their ability to trigger or not the inhibitory functions through the human type II receptors for the Fc region of IgG (FcγRIIB/CD32).

The subset of antibodies or polypeptides selected in step c) for the feature (iv) or (ii), more particularly the subset of antibodies or polypeptides selected to bind specifically or preferentially CD32 (iv) or both CD16 and CD32 (ii) can be further tested and selected for their ability to FcγRIII/CD16 leading to improved ADCC, increased production of cytokines such as Interleukin-2 (IL-2), and/or pro-inflammatory molecules such as Tumor Necrosis Tumor alpha (TNFalpha).

Antibodies of particular interest are those who are able to induce ADCC through the FcgammaRIII and trigger the inhibitory functions through the FcgammaRIIB/CD32.

The functional assays may consist of a calcium mobilization assay and/or a cytokine secretion assay (see FR 0211416 (LFB), the method of which is incorporated herein in the description).

The functional assays may further comprise a specific FcgammaRIII ADCC assay (see W(01/77181 (LFB), the method of which is incorporated herein in the description).
Basically, this specific FcgammaRIII ADCC assay consists of the addition of each antibod or polypeptide to a distinct reaction mixture comprising target cells, effector cells expressin FcγRIII and polyvalent IgG, and the determination of target cells lysis percentage.

Preferably, antibodies or polypeptides according to the invention are selected as displayin one of the following features:
i) bind specifically or preferentially to CD16,
ii) bind to both CD16 and CD32,
iii) do not bind or bind only weakly to both CD16 and CD32, or
iv) bind specifically or preferentially to CD32.
They may be further tested in different culture conditions and different media for improvin yield, antibodies or polypeptide properties as featured in i), ii), iii), or iv) and functions. N binding embraces herein weak binding.

Advantageously, the invention concerns the method as depicted above, wherein antibodie or polypeptides comprising Fc region of human IgG are selected for at least one (fo example two) of the following features:

a)giving a percentage of labeled cells (% positive cells) that represents at least a thirty-fol increase, more preferably a forty-fold increase, as compared to the percentage of cell labeled with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituximab (clinical grade), in an immunofluorescence assay that measures the binding of antibody t CD16 (FcgammaRIIIA) positive indicator cells. An example of this is the R297 antibody Another example is the 4B12 antibody.

b) giving a percentage of labeled cells (% positive cells) that represents at least a ten-fol increase, more preferably a twenty-fold increase, as compared to the percentage of cell labeled with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituximab

(clinical grade), in an immunofluorescence assay that measures the binding of antibody to CD32 (FcgammaRIIB) positive indicator cells. An example of this is the R297 antibody. Another example is the 4B12 antibody.

c) giving a percentage of labeled cells (% positive cells) that represents at least a five-fold decrease, more preferably a ten-fold decrease, as compared to the percentage of cells labeled with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituximab) (clinical grade), in an immunofluorescence assay that measures the binding of antibody to CD16 (FcgammaRIIIA) positive indicator cells. An example of this is the AD1 antibody. Another example is the B11 antibody.

d) giving a percentage of labeled cells (% positive cells) that represents at least a three-fold decrease, more preferably a five-fold decrease, as compared to the percentage of cells labeled with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituximab) (clinical grade), in an immunofluorescence assay that measures the binding of antibody to CD32 (FcgammaRIIB) positive indicator cells. An example of this is the AD1 antibody. Another example is the B11 antibody.

Advantageously, the invention concerns the method as depicted above, wherein antibodies or polypeptides comprising Fc region of human IgG are selected for at least one (for example two) of the following features:

e) giving a mean fluorescence intensity (MFI) value that represents at least a 10-fold increase, more preferably a 15-fold increase, as compared to the MFI value obtained with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituximab) (clinical grade), in an immunofluorescence assay that measures the binding of antibody to CD16 (FcgammaRIIIA) positive indicator cells. An example of this is the R297 antibody. Another example is the 4B12 antibody.

f) giving a mean fluorescence intensity (MFI) value that represents at least a 2-fold increas as compared to the MFI value obtained with equivalent doses of the anti-CD20 monoclon antibody, Rituxan (Rituximab) (clinical grade), in an immunofluorescence assay th measures the binding of antibody to CD32 (FcgammaRIIB) positive indicator cells. A example of this is the R297 antibody. Another example is the 4B12 antibody.

g) a mean fluorescence intensity (MFI) value that represents at least a five-fold decrease, a compared to the MFI value obtained when equivalent doses of anti-CD20 monoclon antibody, Rituxan (Rituximab) (clinical grade), is tested in an immunofluorescence assa that measures the binding of antibody to CD16 positive indicator cells. An example of this i the AD1 antibody. Another example is the B11 antibody.

h) a mean fluorescence intensity (MFI) value that represents at least a five-fold decrease a compared to the MFI value obtained when the same dose of the anti-CD20 monoclon antibody, Rituxan (Rituximab) (clinical grade), is tested in an immunofluorescence assa that measures the binding to CD32 (FcgammaRIIB) positive indicator cells. An example o this is the AD1 antibody. Another example is the B11 antibody.

Advantageously, the invention concerns the method as depicted above, wherein antibodie or polypeptides comprising Fc region of human IgG are selected for one of the followin features:

- an elevated mean fluorescence intensity (MFI) and a strong percentage of labeled cells (% positive cells) in an immunofluorescence assay that measures the binding to CD1 (FcgammaRIII) positive indicator cells. An example of this is the R297 antibody. Anothe example is the 4B12 antibody.

- a low mean fluorescence intensity (MFI) and a low percentage of labeled cells (% positiv cells) in an immunofluorescence assay that measures the binding to CD16 (FcgammaRII

positive indicator cells. An example of this is the AD1 antibody. Another example is the B11 antibody.

- an elevated mean fluorescence intensity (MFI) and a strong percentage of labeled cells (% positive cells) in an immunofluorescence assay that measures the binding to CD32 (FcgammaRIIB) positive indicator cells. An example of this is the R297 antibody. Another example is the 4B12 antibody.

- a low mean fluorescence intensity (MFI) and a low percentage of labeled cells (% positive cells) in an immunofluorescence assay that measures the binding to CD32 (FcgammaRIIB) positive indicator cells. An example of this is the AD1 antibody. Another example is the B11 antibody.

The invention also embraces the purification of antibody glycoforms exhibiting binding properties to FcgammaRIII and FcgammaRII such as those described above by using immobilized soluble forms of FcgammaR (termed FcgammaR-immunoadsorbents) comprising the extracellar regions of the receptors. It makes it possible to enrich for antibodies with an increased or a decreased ability to bind and recruit FcgammaR. Antibodies that efficiently bind FcgammaRIII and/or FcgammaRII can be retained on such FcgammaR-immunoadsorbents, subsequently eluted, allowing the obtention of preparations containing "high" FcgammaR binders antibodies. Conversely, antibodies that poorly bind FcgammaRIII and/or FcgammaRII or that do not bind FcgammaRIII and/or FcgammaRII can be obtained in the effluent fraction of FcgammaR-immunoadsorbents, allowing the obtention of preparations containing "low" FcgammaR binders antibodies.

As referred herein the identified antibodies or polypeptides encompasses the identified cells lines capable of producing said antibodies or polypeptides which display the above relevant features. The invention is directed to a composition comprising at least 80%, 90%, preferably at least 95%, or 99% of antibodies obtainable by the method as depicted above.

Therefore, in another aspect, the invention is aimed at a composition comprising at leas 80%, 90%, preferably at least 95% or 99% of antibodies displaying at least one (for exampl 2) of the following features:

a) a MFI higher than 500, more preferably 700, and a percentage of labeled cells higher tha 90 %, more preferably 95 %, in an immunofluorescence assay that measures the binding o 5, 10, 25 micrograms/ml antibody (containing less than 2% aggregates as determined b HPLC) to $5x10^5$ CD16 (FcgammaRIII) positive indicator cells (characterized by a number o FcgammaRIII receptors lower than 10^5/cell). An example of this is the R297 antibody Another example is the 4B12 antibody.

b) a MFI lower than 80, more preferably 20, and a percentage of labeled cells lower than % , more preferably 2 %, in an immunofluorescence assay that measures the binding of 5 10, 25 micrograms/ml antibody (containing less than 2% aggregates as determined b HPLC) to $5x10^5$ CD16 (FcgammaRIII) positive indicator cells (characterized by a number o FcgammaRIII receptors lower than 10^5/cell). An example of this is the AD1 antibody Another example is the B11 antibody.

c) MFI higher than 50, more preferably 100, and a percentage of labeled cells higher than 8 %, more preferably 90 % in an immunofluorescence assay that measures the binding of 10 25, 50 micrograms/ml antibody (containing less than 2% aggregates as determined b HPLC) to $5x10^5$ CD32 (FcgammaRIIB) positive indicator cells (characterized by a numbe of FcgammaRIIB receptors lower than 5×10^4/cell). An example of this is the R29 antibody. Another example is the 4B12 antibody.

d) a MFI lower than 50, more preferably 20, and a percentage of labeled cells lower than %, more preferably 2 %, in an immunofluorescence assay that measures the binding of 10 25, 50 micrograms/ml antibody (containing less than 2% aggregates as determined b HPLC) to $5x10^5$ CD32 (FcgammaRIIB) positive indicator cells (characterized by a numbe

of FcgammaRIIB receptors lower than 5 x 10^4/cell). An example of this is the AD1 antibody. Another example is the B11 antibody.

The invention is directed to a composition comprising at least 80%, 90 %, preferably at least 95% or 99% of antibodies or peptides displaying the following feature(s) as defined above:

- c);
- d);
- a) and c);
- b) and c);
- b) and d); or
- a) and d).

The invention also embraces the use of a cell line identified in connection with the method as defined above for producing these compositions.

In a still another aspect, the invention relates to the use of a composition as described in c), or b) and c), or a) and c) above to manufacture a medicament for treating auto-immune diseases or other antibody-related pathologies such as allo-immunization.

The invention also relates to the use of a composition as described in c), or b) and c), or a) and c) above to manufacture a medicament for treating allergies and IgE-dependent Type I hypersensitivity.

The invention is also directed to the use of a composition as described in c), or b) and c), or a) and c) above to manufacture a medicament for treating allergies.
Allergies include but are not limited to asthma, allergic rhinitis, allergic sinusitis, anaphylactic syndrome, urticaria, angioedema, atopic dermatitis, allergic contact dermatitis and erythema.

Alternatively, the invention encompasses the use of a composition as described in d), a) ar d) or b) and d) above to manufacture a medicament for treating cancer.

The invention is also directed to the use of a composition as described in in c), or b) and c or a) and c) above to manufacture a medicament for the regulation of hematopoietic ce proliferation dependent on Receptor Tyrosine Kinases (RTKs) by FcgammaRIIB. Fc example, said antibodies are bifunctional molecules such as anti-RTK IgG antibodies wit optimized Fc region capable of efficiently co-aggregating RTKs with FcgammaRIIB, an are used to manufacture a medicament for blocking the proliferation of FcgammaRIIB tumor cells.

The invention further relates to the use of a composition as featured above to manufacture medicament, wherein the antibody recognizes antigens expressed on or bound to the ce surface of target cells. Said antigens include but are not limited to allo-antigens transplantation antigens, self-antigens such as class I and class II Major Histocompatibilit Antigens (HLA), FcgammaRI, FcgammaRIIA, FcgammaRIII molecules, FcalphaR FcepsilonRI, B cell receptor, T cell receptor, tumor antigens such as CD20, Her2/NEU CEA, GD2, allergen such as phospholipase A2, and IgE.

More particularly, the antibodies of the invention recognize Rhesus D antigen t manufacture a medicament for treating or preventing Rhesus alloimmunization of R negative patients, leading to the Hemolytic Disease of the New Born (HDNB).

More particularly, the antibodies of the invention recognize Rhesus D antigen t manufacture a medicament for treating or preventing Idiopathic Thrombocytopenic Purpur (ITP) .

More particularly, the antibodies of the invention recognize HLA Class II molecules t manufacture a medicament for treating or preventing cancers, auto-immune diseases, o graft rejections.

Particular embodiments:

The present invention is to produce and select antibodies with increased or decreased capacity to exert immunomodulatory inhibitory functions through FcgammaRIIB. It relates to the capacity of such antibodies to negatively immunoregulate cells from the immune system or tumor cells, or not. Such a capacity is of major importance in a number of diseases and in various clinical situations such as transplantation, allo-immunization or antibody-based treatments.

More specifically, the present invention relates to the production and selection of antibodies with specific structural characteristics determined by different glycosylation patterns allowing them to turn on or to turn off the negative regulation exerted by FcgammaRIIB on FcgammaRIIB$^+$ cells, such as B cells, monocytes or other antigen-presenting cells (APC), mast cells, basophils, or any other FcgammaRIIB$^+$ cells such as transformed and tumor cells.

The present invention relates to optimized antibodies that will be useful therapeutic tools for controlling antibody responses. It allows to produce and to select antibodies or other molecules optimized for binding to FcgammaRIIB, capable of triggering immunomodulatory inhibitory functions through the recruitment of FcgammaRIIB in antibody-based auto-immune diseases or in unwanted allo-immunization leading to the appearance of pathogenic antibodies, notably against infused recombinant antibodies.

The present invention relates to optimized antibodies that will be useful therapeutic tools for controlling antibody efficacy in antibody-based treatments. It allows to produce and select antibodies with weak or no FcgammaRIIB binding, thus preventing the triggering of immunomodulatory inhibitory functions through FcgammaRIIB during antibody-based therapeutic treatments such as cancer treatments with therapeutic anti-tumor antibodies.

The present invention relates to optimized antibodies that will be useful therapeutic tools fo controlling the recruitment of FcgammaRIIB present on mast cells and basophils b allergen-complexed IgG. As an example, the present invention allows to produce and sele anti-allergen IgG antibodies with an optimized ability to engage FcgammaRIIB and t trigger FcgammaRIIB inhibitory functions through the subsequent co-aggregation c FcgammaRIIB with FcepsilonRI, once IgE are complexed to the same allergen. Anti allergen IgG antibodies with an optimized ability to engage FcgammaRIIB and, hence, t trigger FcgammaRIIB inhibitory functions could represent efficient therapeutic tools in th treatment of allergy. As an example, the present invention allows to produce and select anti FcepsilonRI IgG antibodies with an optimized ability to engage FcgammaRIIB and t trigger FcgammaRIIB inhibitory functions through the subsequent co-aggregation o FcgammaRIIB with FcepsilonRI. Anti-FcepsilonRI IgG antibodies with an optimized abilit to engage FcgammaRIIB and, hence, to trigger FcgammaRIIB inhibitory functions coul represent efficient therapeutic tools in the treatment of allergy.

The present invention relates to optimized antibodies that will be useful therapeutic tools fo triggering the regulation of hematopoietic cell proliferation dependent on Receptor Tyrosin Kinases (RTKs) by FcgammaRIIB. Bifunctional molecules such as anti-RTK IgG antibodie with optimized Fc region capable of efficiently co-aggregating RTKs with FcgammaRIIB could block the proliferation of FcgammaRIIB$^+$ tumor cells.

The present invention relates to optimized antibodies that will be useful therapeutic tools fo controlling the effect of FcgammaRIIB expressed by APC. Examples of such effects include regulation of antigen presentation and T cell recruitment and regulation of acquired immunity.

Binding to FcgammaRIII as used herein refers to a binding of antibodies where only a low percentage of aggregates is present in antibody preparation (<2%), and where antibodies are not complexed on purpose by any mean such as heat or chemical aggregation or by formation of immune-complexes (IC) with the relevant antigen.

Binding to FcgammaRIII as used herein refers to a binding of antibodies complexed with $F(ab)'_2$ anti-human Ig or under immune-complexes (IC) forms with the relevant antigen.

Binding to FcgammaRIIB as used herein refers to a binding of antibodies where only a low percentage of aggregates is present in antibody preparation (<2%), and where antibodies are not complexed on purpose by any mean such as heat or chemical aggregation or by formation of immune-complexes (IC) with the relevant antigen.

Binding to FcgammaRIIB as used herein refers to a binding of antibodies complexed with $F(ab)'_2$ anti-human Ig or under immune-complexes (IC) forms with the relevant antigen.

An activity of antibodies of the present invention includes the inhibition of their binding to FcgammaRIII expressed on indicator cells by antibodies directed to FcgammaRIII.

An activity of antibodies of the present invention includes the inhibition of their binding to FcgammaRIIB expressed on indicator cells by antibodies directed to FcgammaRII.

An activity of antibodies of the present invention includes the inhibition of calcium mobilization, by co-aggregation of such antibodies bound to surface FcgammaRIIB with activating surface receptors such as BCR, TCR, FcepsilonRI, or not. Calcium mobilization as used herein refers to the influx of extracellular calcium into a cell following the recruitment of activating receptors such as BCR.

An activity of antibodies of the present invention includes the inhibition of cytokine production by co-aggregation of such antibodies bound to surface FcgammaRIIB with activating surface molecules such as BCR, or not.

Cytokine production as used herein refers to the release of cytokine by a cell following th recruitment of activating receptors such as BCR.

The inhibition of cytokine production by molecules of the present invention include inhibition of Interleukin-2 (IL-2) production.

The method of the present invention for selecting an immunologically active molecule described below using human IgG antibodies directed against RhD antigen and chimeri mouse/human IgG antibodies directed against HLA-DR class II molecules. As example R297 and AD1 human monoclonal antibodies directed against Rhesus D and 4B12 and B1 chimeric mouse/human antibodies directed against HLA-DR are being used. R297 and 4B1 antibodies are produced in YB2/0 cells (ATCC number CRL-1662). B11 antibody produced by wild-type CHO-DG44 cells. AD1 antibody is produced by cells derived from human B lymphocyte from a donor with anti-RhD antibodies fused with the heteromyelom cell line K6H6/B5 (ATCC number CRL-1823).

The capacity of the purified human or chimeric antibody, either complexed with F(ab) anti-human Ig or not, to bind FcgammaRIIB1 expressed on indicator cells (mouse IIA1 lymphoma B cells expressing a functional mouse BCR and a human recombina FcgammaRIIB1, termed IIA1.6-huFcgammaRIIB1) can be measured by a fluorescen antibody assay.

The capacity of the purified human or chimeric antibody to induce the inhibition of calciu mobilization can be assessed by a calcium measurement assay. Inhibition of calciu mobilization is evaluated by co-aggregating the purified human or chimeric antibody bou to FcgammaRIIB with BCR expressed on indicator cells (mouse IIA1.6 lymphoma B ce expressing a functional mouse BCR and a human recombinant FcgammaRIIB1). It compared to BCR-mediated calcium mobilization induced with $F(ab)'_2$ rabbit anti-goat Ig (RAG) that cross react with mouse IgG.

The capacity of the purified human or chimeric antibody to induce the inhibition of interleukin-2 (IL-2) production can be measured by an enzyme-linked immunosorbent assay (ELISA) that detects and quantifies IL-2. Inhibition of IL-2 production is evaluated by co-aggregating the purified human or chimeric antibody bound to FcgammaRIIB with BCR expressed on indicator cells (mouse IIA1.6 lymphoma B cells expressing a functional mouse BCR and a human recombinant FcgammaRIIB1). It is compared to BCR-mediated IL-2 production induced with F(ab)'$_2$ goat anti-mouse IgG (H+L).

Further details are given below in example 1. Results of these binding and functional tests are presented in the figures.

EXAMPLE 1: Selection of anti-RhD antibodies with improved properties to trigger FcgammaRIIB mediated inhibition.

1. FIGURE LEGENDS, RESULTS AND COMMENTS

Figure 1 represents HPLC profiles of different monoclonal antibodies in order to control the purity and the integrity of the molecules and the absence of polymerized forms : R297 and AD1 anti-RhD antibodies, 4B12 and B11 anti-HLA-DR antibodies.

Name	Retention time (min)	Area	% Area
Peak 1	28.300	72387414	99.04
Peak 2	45.267	700356	0.96

R-297 monoclonal antibody is composed of 99% of monomers.

Figure 2 represents the glycosylation patterns of different antibodies, as determined l capillary electrophoresis. The results are expressed as percentage of the different structur identified. The antibodies were expressed in different cell lines : R297 anti-RhD in YB2 cell line, AD1 anti-RhD in an heterohybridoma cell line, 4B12 anti-HLA-DR in YB2/0 ce line and B11 anti-HLA-DR in CHO-DG44 cell line.

Figure 3 represents the binding of F(ab)'$_2$ goat anti-human IgG (H+L)-complexed R297 a AD1 antibodies to human FcgammaRIIB1 expressed on IIA1.6 cells *as described in [32* The binding of R297 and AD1 human anti-RhD antibodies to human FcgammaRIIB1 assessed by indirect immunofluorescence. Human antibodies (1µg/ml, 5µg/ml, 10µg/ml 25µg /ml ; 50µg /ml) are first complexed with F(ab)'$_2$ goat anti-human IgG (H+L) (at dos of 1.5µg/ml, 7.5µg/ml, 15µg /ml, 40µg/ml, and 80µg /ml, respectively) and then incubat with IIA1.6-huFcgammaRIIB1 cells. Binding of R297 and AD1 antibodies is then reveal with FITC labelled-F(ab)'$_2$ mouse anti-human IgG (H+L) (black curves). The dotted curv show background fluorescence of cells incubated with FITC-F(ab)'$_2$ mouse anti-human Ig (H+L) only.

Results and comments : complexed R297 antibody significantly binds to IIA1. huFcgammaRIIB1 cells even at doses as low as 5µg/ml and 10µg/ml. At these doses, M are 111 and 117, respectively, with more than 70% labelled cells. At higher doses, up 98% cells are stained, with MFI reaching 327 at 50µg/ml. By contrast, no binding of AI antibody to human FcgammaRIIB1 expressed by IIA1.6 cells is observed at a dose 10µg/ml. Only high doses of complexed AD1 antibody bind very weakly to IIA1. huFcgammaRIIB1 cells. Only 9% of FcgammaRIIB1$^+$ IIA1.6 cells are weakly labeled (MF 46) at 50µg/ml. Last, no binding on FcgammaR negative parental IIA1.6 cells is observ with any of the antibodies (not shown). Thus, R297, produced by YB2/0 cells, with a we defined glycosylation pattern, is an example of a FcgammaRIIB "high binder" monoclon antibody, whereas AD1, produced by a cell line obtained by fusing human B cells w heteromyeloma cells (K6H6 x B5), also with a well-defined glycosylation pattern, is example of a FcgammaRIIB "low binder" monoclonal antibody.

Figure 4 represents the binding of F(ab)'$_2$ goat anti-human IgG (H+L)-complexed 4B12 and B11 anti-HLA-DR chimeric mouse/human antibodies to human FcgammaRIIB1 expressed on IIA1.6 cells **as described in [*32*].** The binding of 4B12 and B11 chimeric mouse/human anti-HLA-DR antibodies to human FcgammaRIIB1 is assessed by indirect immunofluorescence.

4B12 and B11 antibodies (1μg/ml, 5 μg/ml, 10μg/ml, 25μg/ml, 50μg/ml) are first complexed with F(ab)'$_2$ goat anti-human IgG (H+L) (at doses of 1.5 μg/ml, 7.5 μg/ml, 15μg/ml, 40μg/ml, and 80μg/ml, respectively) and then incubated with IIA1.6-huFcgammaRIIB1 cells. 4B12 and B11 antibody binding is revealed with FITC labelled-F(ab)'$_2$ mouse anti-human IgG (H+L) (black curves). The dotted curves show background fluorescence of cells incubated with FITC-F(ab)'$_2$ mouse anti-human IgG (H+L) only.

Results and comments : complexed 4B12 antibody significantly binds to IIA1.6-huFcgammaRIIB1 cells at all the doses tested. The percentage of labelled cells is 13 % at a dose of 1 μg/ml and reaches more than 60% at 10 μg/ml. At this dose, a MFI of 103 is observed. No significant binding of B11 antibody to human FcgammaRIIB1 expressed by IIA1.6 cells is observed up to 25 μg/ml. Even at a dose of 50 μg/ml, only 5 % cells are labelled with a MFI of 92. Last, no binding on FcgammaR negative parental IIA1.6 cells is observed with any of the antibodies (not shown). Thus, 4B12, produced by YB2/0 cells, with a well-defined glycosylation pattern, is a second example of a FcgammaRIIB "high binder" monoclonal antibody, whereas B11, produced by CHO-Lec1 cells, also with a well-defined glycosylation pattern, is a second example of a FcgammaRIIB "low binder" monoclonal antibody.

Figure 5 represents the binding of non-complexed R297 and AD1 human anti-RhD antibodies to human FcgammaRIIB1 expressed on IIA1.6 cells **as described in [32].** Less than 2% aggregates are detected in the antibody preparations. R297 and AD1 antibodies (1μg/ml, 5μg/ml, 10μg/ml, 25μg/ml, 50μg/ml) are incubated with IIA1.6-huFcgammaRIIB1 cells. The binding of R297 and AD1 to human FcgammaRIIB1 is then assessed by indirect immunofluorescence. It is revealed with FITC labelled-F(ab)'$_2$ mouse anti-human IgG (H+L)

(black curves). The dotted curves show background fluorescence of cells incubated wit FITC-F(ab)'$_2$ mouse anti-human IgG (H+L) only.

Results and comments : non-complexed R297 antibody significantly binds to IIA1.6 huFcgammaRIIB1 cells even at a low dose such as 5µg/ml (MFI : 28). Up to 86% cells ar labelled at a dose of 50µg/ml, with a MFI reaching 60. No binding of AD1 antibody t human FcgammaRIIB1 expressed by IIA1.6 cells is observed at any dose ranging fro 1µg/ml up to 50µg/ml (percentage of labelled cells <2%, with a MFI of 12 that correspond to the background fluorescence obtained with the secondary revealing FITC labelled-F(ab) mouse anti-human IgG (H+L) Thus, R297 is an example of recombinant antibody produce by YB2/0 cells, with a well-characterized glycosylation pattern, that strongly bind FcgammaRIIB present on cell surface, even under monomeric forms. By contrast, AD antibody is an example of recombinant antibody, produced by a cell line obtained by fusin human B cells with heteromyeloma cells (K6H6 x B5), that does not bind significantl membrane FcgammaRIIB, even when used at doses 10 fold-higher than R297 dose (example: 50µg/ml versus 5µg/ml in the Figure 4)

Figure 6 represents the inhibition of the human anti-RhD antibody R297 binding to huma FcgammaRIIB1 by the mouse AT10 monoclonal antibody directed against huma FcgammaRII. The binding of R297 to human FcgammaRIIB1 is assessed by indire immunofluorescence. IIA1.6-huFcgammaRIIB1 cells are first incubated with the mous mAb AT10 directed against the binding site of human FcgammaRIIA and FcgammaRIIB doses of 0.1µg/ml, 0.5µg/ml, 1µg/ml, or 5µg/ml, and then with 25µg/ml of R297 huma anti-RhD antibody complexed with 40µg/ml F(ab')$_2$ goat anti-human IgG (H+L). Th binding of R297 mAb to human FcgammaRIIB1 is then assessed by indire immunofluorescence using FITC-F(ab)'$_2$ mouse anti-human IgG (H+L) as revealin antibodies (black curves). The dotted curves show background fluorescence of cel incubated with FITC-F(ab)'$_2$ mouse anti-human IgG (H+L) only.

Results and comments : a dose-dependent inhibition of the binding of R297 mAb to surfa FcgammaRIIB is achieved when the mAb AT10 directed against the binding site FcgammaRIIB is used as competitor. As little as 0.1µg/ml AT10 allows to decrease the MF

down from 420 to 232. When 5 μg/ml AT10 are used, only a marginal binding of R297 to FcgammaRIIB IIA1.6 cells is observed (% labeled cells: 11%, MFI: 59). It indicates that the binding of R297 antibody to FcgammaRIIB$^+$ IIA1.6 cells is mediated through the FcgammaRIIB expressed on these cells.

Figure 7 represents the inhibition of the human anti-RhD antibody R297 binding to FcgammaRIIB1$^+$ IIA1.6 cells by F(ab)'$_2$ mouse anti-human FcgammaRII mAb, 6C4. IIA1.6-huFcgammaRIIB1 cells are first incubated with F(ab)'$_2$ 6C4 mAb directed against the binding site of human FcgammaRII at doses of 1μg/ml, 10μg/ml, 25μg/ml, 50μg/ml or 100μg/ml, and then with 25μg/ml of R297 complexed with 40μg/ml F(ab')$_2$ goat anti-human IgG (H+L). The binding of R297 mAb to human FcgammaRIIB1 is then assessed by indirect immunofluorescence using FITC-F(ab)'$_2$ mouse anti-human IgG(H+L) as revealing antibodies (black curves). The dotted curves show background fluorescence of cells incubated with FITC-F(ab)'$_2$ mouse anti-human IgG (H+L) only.
Results and comments : a dose-dependent inhibition of the binding of R297 mAb to surface FcgammaRIIB is achieved when the F(ab)'$_2$ 6C4 mAb directed against the binding site of FcgammaRIIB is used as competitor. 10μg/ml F(ab)'$_2$ 6C4 allow to decrease the MFI down from 420 to 131. When 100μg/ml F(ab)'$_2$ 6C4 are used, only a marginal binding of R297 to FcgammaRIIB IIA1.6 cells is observed (% labeled cells: 7%, MFI: 68). This dose-dependent inhibition obtained with a F(ab)'$_2$ fragment of a monoclonal antibody directed against the FcgammaRII binding site indicates that the binding of R297 antibody to FcgammaRIIB$^+$ IIA1.6 cells is mediated through the FcgammaRIIB expressed on these cells.

Figure 8 represents the inhibition of the binding of the chimeric mouse/human anti-HLA-DR antibody 4B12 to FcgammaRIIB1$^+$ IIA1.6 cells by mouse AT10 monoclonal antibody and by mouse F(ab)'$_2$ 6C4 monoclonal antibody, both directed against the binding site of human FcgammaRII. IIA1.6-huFcgammaRIIB1 cells are first incubated with AT10 **(A)** or F(ab)'$_2$ 6C4 **(B)** mAbs at doses of 0.1μg/ml, 0.5μg/ml, 1μg/ml for AT10, or 1μg/ml, 10μg/ml, 25μg/ml for F(ab)'$_2$ 6C4. IIA1.6-huFcgammaRIIB1 cells are then incubated with 25μg/ml of 4B12 complexed with 40μg/ml F(ab')$_2$ goat anti-human IgG (H+L). The binding

of 4B12 mAb to human FcgammaRIIB1 is then assessed by indirect immunofluorescen using FITC-F(ab)'$_2$ mouse anti-human IgG(H+L) as revealing antibodies (black curves). Tl dotted curves show background fluorescence of cells incubated with FITC-F(ab)'$_2$ mou anti-human IgG (H+L) only.

Results and comments : a strong inhibition of the binding of 4B12 mAb to surfa FcgammaRIIB is achieved when the AT10 and the F(ab)'$_2$ 6C4 mAbs directed against tl binding site of FcgammaRIIB are used as competitors. One µg/ml F(ab)'$_2$ 6C4 leads to 50% inhibition of 4B12 binding (% labelled cells : from 43% to 22% ; MFI: from 113 · 76). 10µg/ml F(ab)'$_2$ 6C4 totally inhibits the binding of 4B12 (percentage of labelled cell 2% down from 43% ; MFI down from 113 to 36). Similarly, a strong inhibition of th binding of 4B12 mAb to surface FcgammaRIIB is achieved even at low doses of AT1 mAb. As little as 0.1µg/ml AT10 allows to decrease the MFI down from 113 to 76 and th percentage of cells down from 43% to 22%. When 0.5µg/ml AT10 is used, only a margin binding of 4B12 to FcgammaRIIB IIA1.6 cells is observed (% labeled cells: 3%, MFI: 109 1µg/ml AT10 completely blocks the binding of 4B12 to FcgammaRIIB IIA1.6 cells (MF 12 ; % labelled cells: <2%). Thus, as observed with the anti-RhD 297 antibody, the bindi of the anti-HLA-DR 4B12 antibody, produced by YB2/0 cells, to FcgammaRIIB IIA1 cells is mediated through the FcgammaRIIB expressed on these cells. It indicates th recombinant antibodies produced by YB2/0 cells exhibit a strong binding to FcgammaRI expressed on surface cells, whatever their specificities.

Figure 9 represents a comparison of the binding of human R297 and AD1 anti-Rh monoclonal antibodies to human FcgammaRIIIA expressed by stably transfected Jurkat ce (Jurkat-huFcγRIIIA/gamma) with the binding of the chimeric mouse/human anti-CD Rituxan (rituximab) monoclonal antibody. In these experiments, antibodies are n aggregated and are not bound to their specific antigen. 5 x 10^5 Jurkat-huFcγRIIIA/gamm cells are first incubated with the various mAbs used at doses of 5µg/ml, 10µg/ml 25µg/ml. The binding of monoclonal antibodies to human FcgammaRIIIA is then assess by indirect immunofluorescence using FITC-F(ab)'$_2$ mouse anti-human IgG(H+L) revealing antibodies. Flow cytometry acquisition is performed with a FACScan Calib

(Becton Dickinson, Mountain View, California, USA), and analysis is performed with the Cell Quest Pro software. Percentage of positive cells (A) and Mean Fluorescence Intensity (MFI) (B) are calculated after acquisition and analysis of ten thousand events for each condition. The value of background MFI obtained with the revealing FITC-F(ab)'$_2$ mouse anti-human IgG(H+L) antibodies is 12.

Results and comments : Panel A and panel B show that three patterns of binding are observed depending on the monoclonal antibody tested. The anti-RhD R297 monoclonal antibody binds more than 90% of FcgammaRIIIA positive Jurkat cells whatever the doses used (5µg/ml, 10µg/ml and 25µg/ml). It is considered therefore as a high binder, with a MFI ranging from around 500 up to 700 (panel B). By contrast, the anti-CD20 monoclonal antibody Rituxan binds only weakly to FcgammaRIIIA positive Jurkat cells ; around 30% cells are labeled when the Rituxan antibody is used at the higher dose (25µg/ml) ; only 10% cells are labeled with 10µg/ml of Rituxan, while there is no significant labeling when the antibody is tested at 5µg/ml (panel A). In addition, a low MFI is observed whatever the dose of Rituxan tested (i.e., <100) (panel B). Moreover, the monoclonal antibody AD1, directed against RhD, shows a third pattern of binding to FcgammaRIIIA Jurkat cells. This antibody binds a very low percentage of cells at any dose tested (panel A) (<5%) with a low MFI (<70) at each dose. Thus, three groups of monoclonal antibodies with Fc region from the human IgG1 subclass can be defined using this assay that uses antibody preparations with no or very few aggregates (less than 2% as determined by HPLC) : firstly, "high" mAb binders that bind a high percentage of FcgammaRIIIA positive cells (>90%) with an elevated MFI (>500), even at a low dose (5µg/ml) ; secondly, "intermediate" mAb binders that bind significantly FcgammaRIIIA positive cells only at the higher dose of antibody tested (about 30% labelled cells at 25µg/ml), whereas lower doses show only a marginal binding. These "intermediate" mAb binders label cells with a low MFI (<100) whatever the dose tested ; thirdly, "low" mAb binders that bind less than 5% cells, with a low MFI (<70), whatever the dose tested.

Figure 10 represents a comparison of the binding of human R297 and AD1 anti-RhD monoclonal antibodies to human FcgammaRIIB1 expressed by IIA.1.6 cells (IIA1.6-

huFcgammaRIIB1 cells) with the binding of the chimeric mouse/human anti-CD20 Rituxa (rituximab) monoclonal antibody. In these experiments, antibodies are not aggregated an are not bound to their specific antigen. 5 x 10^5 IIA1.6-huFcgammaRIIB1 cells are fir incubated with the various mAbs used at doses of 10µg/ml, 25µg/ml or 50µg/ml. Th binding of monoclonal antibodies to human FcgammaRIIB1 is then assessed by indire immunofluorescence using FITC-F(ab)'$_2$ mouse anti-human IgG(H+L) as revealin antibodies. Flow cytometry acquisition is performed with a FACScan Calibur (Becto Dickinson, Mountain View, California, USA), and analysis is performed with the Cell Que Pro software. Percentage of positive cells (A) and Mean Fluorescence Intensity (MFI) (B are calculated after acquisition and analysis of ten thousand events for each condition. Th value of background MFI obtained with the revealing FITC-F(ab)'$_2$ mouse anti-huma IgG(H+L) antibodies is 12.

Results and comments : Panel A and panel B show that three patterns of binding a observed depending on the monoclonal antibody tested. The anti-RhD R297 monoclon antibody binds more than 50% IIA1.6-huFcgammaRIIB1 cells at a dose of 10µg/ml an more than 80% at doses of 25µg/ml and 50µg/ml. It is considered therefore as a hig FcgammaRIIB binder, with a MFI reaching more than 120 at a dose of 50µg/ml (panel B By contrast, the anti-CD20 monoclonal antibody Rituxan binds only weakly to IIA1.6 huFcgammaRIIB1 cells ; around 25% cells are labeled when the Rituxan antibody is used a the higher dose (50µg/ml) ; less than 5% cells are labeled with 25µg/ml of Rituxan, whi there is no significant labeling when the antibody is tested at 10µg/ml (panel A). In additio a low MFI is observed whatever the dose of Rituxan tested (<60) (panel B). Moreover, th monoclonal antibody AD1, directed against RhD, shows a third pattern of binding to IIA1.6 huFcgammaRIIB1 cells. This antibody does not bind the cells when tested at 10µg/ml an 25µg/ml and binds a very low percentage of cells at 50µg/ml (panel A) (<5%) with a lo MFI at this dose (<65). Thus, three groups of monoclonal antibodies with Fc region fro the human IgG1 subclass can be defined using this assay that uses antibody preparatio with no or very few aggregates (less than 2% as determined by HPLC) : firstly, "high" mA binders that bind a high percentage of huFcgammaRIIB1 positive cells (>80%) with a elevated MFI (>70), at doses of 25µg/ml and 50µg/ml ; secondly, "intermediate" mA

binders that bind significantly huFcgammaRIIB1 positive cells only at the higher dose of antibody tested (about 25% labeled cells at 50µg/ml), whereas lower doses show only a marginal binding, if any. These "intermediate" mAb binders label huFcgammaRIIB1 positive cells with a low MFI (<60) whatever the dose tested ; thirdly, "low" mAb binders that do not bind huFcgammaRIIB1 positive cells at doses of 10µg/ml and 25µg/ml, and that bind less than 5% cells, with a low MFI (<70), at a dose of 50µg/ml.

Figure 11 summarizes the binding data (% labeled cells and Mean Fluorescence Intensity, MFI) obtained with the human R297 and AD1 anti-RhD monoclonal antibodies and with the chimeric mouse/human anti-CD20 Rituxan (rituximab) monoclonal antibody when FcgammaRIIIA positive Jurkat cells and IIA1.6-huFcgammaRIIB1 positive cells are tested in indirect immunofluorescence assays (see legends to Figures 9 and 10). Percentage of positive cells are represented in ordinate and MFI are represented in abscissa. Upper and lower left panels indicate % and MFI values obtained with R297, AD1 and Rituxan (rituximab) monoclonal antibodies. Upper and lower right panels indicate % and MFI values obtained with AD1 and Rituxan (rituximab) monoclonal antibodies using a different scale in ordinate and abscissa.

Results and comments: three groups of monoclonal antibodies with Fc region from the human IgG1 subclass can be defined using this indirect immunofluorescence assay with antibody preparations that contain no or very few aggregates (less than 2% as determined by HPLC) : i) antibodies that give rise to an elevated MFI and to a high percentage of labeled cells (>80%) at various doses (FcgammaRIIIA, 5, 10, 25µg/ml ; FcgammaRIIB1, 10, 25, 50µg/ml) such as the R297 monoclonal antibody (FcgammaRIIIA: upper left panel ; FcgammaRIIB1: lower left panel) ; ii) antibodies that give rise to an intermediate MFI and to an intermediate percentage of labeled cells (20-30%) only at a high dose (FcgammaRIIIA, 25µg/ml ; FcgammaRIIB1, 50µg/ml), such as the Rituxan monoclonal antibody (FcgammaRIIIA: upper right panel ; FcgammaRIIB1: lower right panel, grey symbols) ; iii) antibodies that give rise to an intermediate MFI and to a low percentage of labeled cells (<5-10%) only at a high dose (FcgammaRIIIA, 25µg/ml ; FcgammaRIIB1, 50µg/ml), such as the

AD1 monoclonal antibody (FcgammaRIIIA : upper right panel ; FcgammaRIIB1 : low
right panel, open symbols).

Figure 12 represents the inhibition of calcium mobilization by R297 human anti-Rh
antibody. IIA1.6-huFcgammaRIIB1 cells are stimulated either with F(ab)'$_2$ rabbit anti-go
IgG (RAG) (10µg/ml) that cross-react with mouse Ig or with R297 antibody (100µg/ml)
presence of F(ab)'$_2$ goat anti-human IgG (H+L) (GAH) (10µg/ml) and F(ab)'$_2$ rabbit ant
goat IgG (RAG) (10µg/ml). This latter assay allows to efficiently cross-link murine surfac
IgG with the human FcgammaRIIB1 expressed by IIA1.6-huFcgammaRIIB1 cells. The Ca
influx observed in this condition of BCR/ FcgammaRIIB1 cross-linking is compared
BCR-mediated Ca^{2+} influx induced with F(ab)'$_2$ RAG (10µg/ml). Intracellular free Ca
levels in Fluo-3 AM loaded cells are monitored by flow cytometry. Ca^{2+} release fro
intracellular Ca^{2+} stores is measured in presence of 1mM EGTA (left part of the curves), ar
Ca^{2+} entering the cells is measured after addition of 6mM $CaCl_2$ (right part of the curves).
Results and comments : the addition of F(ab)'$_2$ rabbit anti-goat IgG (RAG) antibodi
triggers calcium release from the intracellular stores (left peak of the curve, dotted line)
well as a calcium influx into IIA1.6-huFcgammaRIIB1 cells (right peak of the curve, dott
line). A strong inhibition of the calcium influx (right peak of the curve, black line),
characteristics of the engagement of FcgammaRIIB on B cells, is obtained by incubatir
IIA1.6-huFcgammaRIIB1 cells with R297 antibody (100µg/ml) in presence of F(ab)'$_2$ go
anti-human IgG (H+L) (GAH) (10µg/ml) and F(ab)'$_2$ rabbit anti-goat IgG (RAG) (10µg/m
As expected when the FcgammaRIIB inhibitory function is triggered, no inhibition of t
calcium release from intracellular stores is observed (left peak of the curve, black line
Thus, this experiment shows that the R297 monoclonal antibody is capable of triggerir
inhibitory functions mediated through FcgammaRIIB expressed on lymphoma B cells,
triggering which is known to lead to the blockade of B cell activation and differentiation in
antibody-producing cells.

Figure 13 represents the inhibition of IL-2 production by R297 but not by AD1 human an
RhD antibodies . 5 x 10^5 IIA1.6-huFcgammaRIIB1 cells are stimulated for 22h either wi

50µg/ml F(ab)'$_2$ rabbit anti-goat IgG (RAG) alone or with 50µg/ml F(ab')$_2$ rabbit anti-goat IgG and R297 or AD1 antibody at different concentrations as indicated in abscissa (from 1 to 100µg/ml). The RAG F(ab)'$_2$ fragments cross-react with both murine and human IgG, allowing to cross-link the mouse surface IgG expressed by IIA1.6 huFcgammaRIIB1 positive cells and the human monoclonal IgG antibody bound to FcgammaRIIB1. The production of mouse IL-2 by IIA1.6-huFcgammaRIIB1 cells in cell culture supernatants is measured by an ELISA assay using 1µg/ml rat anti-mouse IL-2 as capture antibody and 1µg/ml biotinylated rat anti-mouse IL-2 as detection antibody.

Results and comments: the stimulation of IIA1.6-huFcgammaRIIB1 positive cells that express surface IgG by F(ab)'$_2$ rabbit anti-goat IgG (RAG) cross-reacting with mouse IgG triggers the secretion of IL-2 (about 380 pg/ml after 22h of culture of 5 x 10^5 IIA1.6 FcgammaRIIB1 cells). Strikingly, the presence of various doses of R297 antibody (from 1µg/ml to 100µg/ml) in presence of RAG F(ab)'$_2$ fragments cross-reacting with both murine and human IgG induces a dose-dependent inhibition of IL-2 secretion. As low as 1µg/ml R297 antibody induces about a 30% inhibition of IL-2 production, while 50µg/ml induces up to 50% inhibition. By contrast, AD1 antibody does not inhibit IL-2 production by IIA1.6-huFcgammaRIIB1 cells cultured in presence of RAG F(ab)'$_2$ fragments, even when used at a high dose such as 100µg/ml (<3% decrease). Thus, this experiment shows that the R297 monoclonal antibody, a high mAb binder to FcgammaRIIB1 expressed by IIA1.6-huFcgammaRIIB1 cells, is capable of triggering inhibitory functions mediated through FcgammaRIIB expressed on lymphoma B cells. Notably, it leads to the blockade of IL-2 production by these cells. In addition, this experiment also shows that the AD1 monoclonal antibody, a low mAb binder to FcgammaRIIB1 expressed by IIA1.6-huFcgammaRIIB1 cells, is not able to trigger inhibitory functions mediated through FcgammaRIIB expressed on lymphoma B cells.

EXAMPLE 1: Selection of anti-RhD antibodies with improved properties to trigger FcgammaRIIB mediated inhibition.

Material and methods

Cell lines and antibodies

The mouse IIA1.6 B cell lymphoma is a FcgammaR-defective variant of A20 B cells (Jor *et al.*, 1986) that bears a deletion of the 5' end of the FcgammaRII gene (Lewis et al., 198 Bonnerot *et al.*, 1991) and does not transcribe the genes encoding the α- and the γ-chains mouse FcgammaRIII (Bonnerot *et al.*,1991). IIA.1.6 cells were transfected by the cDN encoding FcgammaRIIB1 obtained from Dr. M. Hogarth (Melbourne University, Parkvil Victoria, Australia) (Hogarth *et al.*, 1987). IIA1.6 and IIA1.6-huFcgammaRIIB1 cells we cultured in Click medium [RPMI 1640 (GIBCO, Paisley, Scotland) supplemented with 10 heat-inactivated FCS (Hyclone Laboratories Inc., Logan, Utah, USA), 100U/ml penicilli 100μg/ml streptomycin, 2mM L-glutamine, 5mM sodium pyruvate, 0.5μM 2-mercaptoethanol.

The AD1 B cell line was obtained by fusing human B lymphocytes from a donor immunized with human Rh positive recd blood cells with the heteromyeloma cell line K6H6/B5 (ATCC number CRL-1823) and subsequent cloning was performed by limiting dilutions. AD1 cells were cultured in RPMI 1640 medium supplemented with 5% FCS.

The YB2/0 cell line (ATCC number CRL-1662) was used to produce a human recombinant anti-Rh D (R297) monoclonal antibody and a chimeric anti-HLA-DR (4B12) recombinant monoclonal antibody. YB2/0 transfected cells were cultured in a specific EM culture medium with 5% of FCS.

CHO DG44 cells (ATCC number CRL-1735) were cultured in a specific EM culture medium supplemented with 5% of FCS to produce the chimeric anti-HLA-DR (B11) recombinant antibody.

All the producing cell lines were adapted and cultured in a protein free culture medium and the production were performed in rolling bottles or in fermentors. After the production, the monoclonal antibodies were purified by using protein-A affinity chromatography. The purified antibodies were then stored frozen at –20°C.

Rituxan (Rituximab) (Genentech, South San Francisco, California, USA) is a chimeric mouse/human anti-CD20 monoclonal antibody used in the treatment of patients with relapsed or refractory low-grade or follicular, $CD20^+$, B-cell non -Hodgkin's lymphoma (NHL). Commercial Rituxan vials are stored at 2°C to 8°C in a sterile preservative-free solution at a concentration of 10mg/ml.

Immunofluorescence assay

First, recombinant human antibodies were complexed under polymeric forms for 30 min with affinity-purified goat $F(ab)'_2$ anti-human IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, Pennsylvania, USA). $5x10^5$ indicator cells were then incubated for 30 min with complexed antibodies in ice-cold phosphate-buffered saline containing 0.5% bovine serum albumin (PBS-BSA). Cells were then washed with PBS-BSA, and antibody binding was detected by incubation with FITC-labelled mouse $F(ab)'_2$ anti-human IgG (H+L) (Jackson ImmunoResearch) for 30 min on ice. After further washing, flow cytometry analysis was performed with a FACScalibur 4CA (Becton Dickinson, Mountain View, California, USA), using the Cell Quest Pro software.

Facscalibur 4CA technical specifications

A 15mW, 488nm air cooled Argon-ion laser is used for acquisition of FITC fluorochrome. Estimated detection limit is 200 FITC equivalent molecules per particle. Logarithmic amplifiers for FL1 (FITC) provide four log decade range. Sorting speed is 300cells/sec and the sorting purity is more than 95%.

Calcium mobilization assay

IIA1.6-huFcgammaRIIB1 cells were incubated or not with R297 or AD1 mAb for 30 min on ice. 10^6 cells were incubated with 5mM Fluo-3 AM (Molecular Probes, Eugene, Oregon, USA) for 30 min at room temperature in RPMI 1640 containing 0.2% Pluronic F-127 (Sigma Chemicals Co., St. Louis, Missouri, USA). The loaded cells were then washed three times with RPMI 1640 and adjusted to 10^6/ml. Cells were stimulated with rabbit $F(ab')_2$ anti-

goat IgG (H+L) (RAG) (Jackson ImmunoResearch) alone or rabbit F(ab)'$_2$ anti-goat Ig((H+L) (RAG) and goat F(ab)'$_2$ anti-human IgG (H+L) (GAH) (Jackson ImmunoResearch) i RPMI 1640 containing 1mM EGTA. After 150 sec of stimulation, 6mM $CaCl_2$ were adde Intracellular calcium mobilization was detected by flow cytometry, performed with FACScalibur 4CA, using the Cell Quest Pro software. Intracellular calcium concentratio means were then calculated with the FCS assistant 1.2.9 beta software (Becton Dickinson).

Detection of IL-2 production

10^5 IIA1.6-huFcgammaRIIB1 cells were stimulated for 18 h with F(ab)'$_2$ rabbit anti-go (Jackson ImmunoResearch) alone or with F(ab')$_2$ rabbit anti-goat and R297 or AD1. Th presence of mouse IL-2 was measured by ELISA assay, using 1µg/ml rat anti-mouse IL- (Pharmingen) as capture antibody and 1µg/ml biotinylated rat anti-mouse IL-2 (Pharminge as detection antibody.

Binding studies on soluble Fc fragments

Antibody interaction with the Fc receptor is the primary event needed for cell regulation. However, cells or cell ghosts expressing are not so easy to use "in vitro" and require either negative control (not always available) or inhibition studies, to insure the binding specificity. To overcome these problems, recombinant soluble Fc receptor were used to evaluate the binding capacity of monoclonal or polyclonal antibodies to Fc receptor. Two types of experiments were performed :

- bindind studies in the absence of antigens, in this condition, the data were assessed by flow cytometry by measuring MFI
- binding studies in the presence of antigens : the data were assessed by using immunoenzymatic tests in which antigen or antigen bearing membranes were coated onto microplates.

Following described method was used for anti-D antibodies using Rhesus positive red cells. However, it could be extended to other antigens, by using the appropriate cell carrying onto its cell membrane the specific antigen.

Binding assays with anti-RhD antibodies were performed as following :

1- Rhesus positive red cells were traited with papaïne (0.1mg/ml) for 10 minutes at 37°C. After washing, red cells were diluted in cold CFC buffer (0,1M glucose; 0,07M NaCl; 0,02M borique acid; 0,035M tri sodium citrate; 0,002M tétra-sodium EDTA). Papaïne traited red cells (10^7 per ml) were incubated for 2 minutes in a P96 microtiter U well plate assay (100µl/well). Plates were spun (2 minutes, 1000rpm), supernatant was removed then 100µl glutaraldehyde solution (0.375% in cold PBS) was added for 10 minutes. Finally, plates were spun (2 minutes, 1000rpm) and washed with PBS

2- Anti-D antibodies were added and incubated for 1h at room temperature and the plate were washed with a buffer solution.

3- Recombinant soluble Fc receptors were then added. After an 1h incubation period, the plates were washed. The soluble receptors, FcRIIB (CD32) and FcRIIIA (CD16) were produced as a fusion protein by coupling to the GST. The recombinant receptors were purified by affinity chromatography on glutathion agarose.

PUC119-derived vector containing the FcgRIIA2 cDNA and pcDNA3 vector containing cDNA encoding the FcgRIIIB (NA2 allele) ectodomains were used to construct cDNA encoding recombinant GST fused to the extracellular (EC) region of FcgRIIA (GST-ECIIA) or FcgRIIIB (GST-ECIIIB). A two step PCR was performed for generating the GST-ECIIA construct. The following amplimers were used for this step : 5'-GCAGCTCCCCCAAAGGCTGTG-3' (sense) and 5'-TTGGACAGTGATGGTCACAGG-3' (antisense). The second step allowed the addition of restriction sites to the PCR product for cloning into the pGEX-2T expression vector (Amersham-Pharmacia Biotech, England). For cloning FcgammaRIIIB EC region, one step PCR was performed using sense (5'-TGGATGAATTCCCTATTAAGTGATGGTGATGTT-3') and antisense (5'-ATCGGATCCCGACTGAAGATCTC-3') amplimers containing EcoRI and BamH1restriction sites that made possible the cloning of the cDNA into pGEX-2T vector. Fusion proteins (GST-ECIIA, GST-ECIIIB) were purified by affinity chromatography onto glutathione agarose beads according to the manufacturer's instructions.

4- The binding of the soluble receptor is assessed by adding an antibody labeled with biotine or alkaline phosphatase and specific of the studied receptor. This antibody did not inhibit the binding of the Fc to his receptor. A second anti-IgG and labeled monoclonal antibody is added and the OD are registered with a spectrophotometer.

5

CLAIMS

1. A method for the preparation of human, humanized or chimæric antibodies or polypeptides comprising Fc region of human IgG with enhanced therapeutic properties and decreased side effects, wherein said method comprises the step consisting of :

a) providing candidate human, humanized or chimæric antibodies or polypeptides comprising Fc region of human IgG produced from different cell lines selected from hybridoma, heterohybridoma, animal cell lines or eukaryote microorganisms, transfected with a vector comprising the coding sequence for said antibody or polypeptide,

b) testing the binding of said antibodies or polypeptides on Fcgamma receptors including CD16 (FcgammaRIII) and CD32 (FcgammaRIIA and B),

c) selecting antibodies or polypeptides which :

i) bind specifically or preferentially to a first Fcgamma receptor,

ii) bind to both said first Fcgamma receptor and a second Fcgamma receptor,

iii) do not bind or bind only weakly to both said first Fcgamma receptor and said second Fcgamma receptor, or

iv) bind specifically or preferentially to said second Fcgamma receptor.

2. A method according to claim 1, wherein step c) consists of selecting antibodies or polypeptides which:

i) bind specifically or preferentially to CD16,

ii) bind to both CD16 and CD32,

iii) do not bind or bind only weakly to both CD16 and CD32, or

iv) bind specifically or preferentially to CD32.

3. A method according to claim 1 or 2 which further comprise testing the binding to a third or fourth Fcgamma receptor.

4. A method according to one of claims 1 to 3, wherein the binding assays is perform using:

i) indicator cells from cell lines that express different Fc receptors on their cell surface, or

ii) recombinant Fc receptors comprising FcgammaR ectodomains, Fc receptors derive peptides, or

iii) in an assay reaction mixture comprising a soluble Fc receptors or fragment thereof.

5. A method according to claim 4, wherein one of the Fc receptors is FcgammaRIIB.

6. A method according to claim 4, wherein one of the Fc receptors is FcgammaRIII.

7. A method according to one of claims 1 to 6, wherein the subset of antibodies polypeptides selected in step c) for the feature (iv) or (ii), more particularly the subset antibodies or polypeptides selected to bind specifically or preferentially CD32 (iv) or bo CD16 and CD32 (ii), are further tested and selected for their ability to trigger or not t inhibitory functions through the human type II receptors for the Fc region of Ig (FcγRIIB/CD32).

8. A method according to one of claims 1 to 6, wherein the subset of antibodies polypeptides selected in step c) for the feature (iv) or (ii), more particularly the subset antibodies or polypeptides selected to bind specifically or preferentially CD32 (iv) or bo CD16 and CD32 (ii), are further tested and selected for their ability to trigger FcγRIII/CD1 leading to improved ADCC, increased production of cytokines such as Interleukin-2 (IL-2 and/or pro-inflammatory molecules such as Tumor Necrosis Tumor alpha (TNFalpha).

9. A method according to claim 7 or 8 wherein said subset of antibodies or polypeptides a selected for their ability to induce ADCC through the FcgammaRIII and trigger t inhibitory functions through the FcgammaRIIB/CD32.

10. A method according to one of claims 7 to 9, wherein the functional assays consist of a calcium mobilization assay and/or a cytokine secretion assay.

11. A method according to one of claims 7 to 10, wherein the functional assays further comprise a specific FcgammaRIII ADCC assay.

12. A method according to claim 11, wherein the specific FcgammaRIII ADCC assay consists of the addition of each antibody or polypeptide to a distinct reaction mixture comprising target cells, effector cells expressing FcγRIII and polyvalent IgG, and the determination of target cells lysis percentage.

13. A method according to one of claims 1 to 12, wherein said antibodies or polypeptides are selected as displaying one of the following features:
i) bind specifically or preferentially to CD16,
ii) bind to both CD16 and CD32,
iii) do not bind or bind only weakly to both CD16 and CD32, or
iv) bind specifically or preferentially to CD32,
are further tested in different culture conditions and different media for improving yield, antibodies or polypeptide properties as featured in i), ii), iii), or iv) and functions.

14. A method according to one of claims 1 to 13, wherein antibodies or polypeptides comprising Fc region of human IgG are selected for at least one of the following features:

a) giving a percentage of labeled cells (% positive cells) that represents at least a thirty-fold increase, more preferably at least a forty-fold increase, as compared to the percentage of cells labeled with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituximab) (clinical grade), in an immunofluorescence assay that measures the binding of antibody to CD16 (FcgammaRIIIA) positive indicator cells;

b) giving a percentage of labeled cells (% positive cells) that represents at least a ten-fold increase, more preferably a twenty-fold increase, as compared to the percentage of cells

labeled with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituxima (clinical grade), in an immunofluorescence assay that measures the binding of antibody CD32 (FcgammaRIIB) positive indicator cells;

c) giving a percentage of labeled cells (% positive cells) that represents at least a five-fo decrease, more preferably a ten-fold decrease, as compared to the percentage of cells label with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituximab) (clinic grade), in an immunofluorescence assay that measures the binding of antibody to CD (FcgammaRIIIA) positive indicator cells;

d) giving a percentage of labeled cells (% positive cells) that represents at least a three-fo decrease, more preferably a five-fold decrease, as compared to the percentage of ce labeled with equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituxima (clinical grade), in an immunofluorescence assay that measures the binding of antibody CD32 (FcgammaRIIB) positive indicator cells.

15. A method according to one of claims 1 to 14, wherein antibodies or polypeptid comprising Fc region of human IgG are selected for at least one of the following features:

e) giving a mean fluorescence intensity (MFI) value that represents at least a 10-fo increase, more preferably a 15-fold increase, as compared to the MFI value obtained w equivalent doses of the anti-CD20 monoclonal antibody, Rituxan (Rituximab) (clinic grade), in an immunofluorescence assay that measures the binding of antibody to CD (FcgammaRIIIA) positive indicator cells;

f) giving a mean fluorescence intensity (MFI) value that represents at least a 2-fold increa as compared to the MFI value obtained with equivalent doses of the anti-CD20 monoclo antibody, Rituxan (Rituximab) (clinical grade), in an immunofluorescence assay th measures the binding of antibody to CD32 (FcgammaRIIB) positive indicator cells;

g) a mean fluorescence intensity (MFI) value that represents at least a five-fold decrease, as compared to the MFI value obtained when equivalent doses of anti-CD20 monoclonal antibody, Rituxan (Rituximab) (clinical grade), is tested in an immunofluorescence assay that measures the binding of antibody to CD16 positive indicator cells;

h) a mean fluorescence intensity (MFI) value that represents at least a five-fold decrease as compared to the MFI value obtained when the same dose of the anti-CD20 monoclonal antibody, Rituxan (Rituximab) (clinical grade), is tested in an immunofluorescence assay that measures the binding to CD32 (FcgammaRIIB) positive indicator cells.

16. A method according to one of claims 1 to 15, wherein antibodies or polypeptides comprising Fc region of human IgG are selected for at least one of the following features:

- an elevated mean fluorescence intensity (MFI) and a strong percentage of labeled cells (% positive cells) in an immunofluorescence assay that measures the binding to CD16 (FcgammaRIII) positive indicator cells.

- a low mean fluorescence intensity (MFI) and a low percentage of labeled cells (% positive cells) in an immunofluorescence assay that measures the binding to CD16 (FcgammaRIII) positive indicator cells.

- an elevated mean fluorescence intensity (MFI) and a strong percentage of labeled cells (% positive cells) in an immunofluorescence assay that measures the binding to CD32 (FcgammaRIIB) positive indicator cells.

- a low mean fluorescence intensity (MFI) and a low percentage of labeled cells (% positive cells) in an immunofluorescence assay that measures the binding to CD32 (FcgammaRIIB) positive indicator cells.

17. A method of purification of antibodies exhibiting binding properties to FcgammaRI and FcgammaRII such as those defined in one of claims 14 to 16 comprsing a ste consisting of enriching said antibodies by using immobilized soluble forms of Fcgammal termed FcgammaR-immunoadsorbents, comprising the extracellar regions of the receptors.

18. A method according to claim 17 to enrich for antibodies that efficiently bir FcgammaRIII and/or FcgammaRII, wherein said method comprises the retention antibodies on said FcgammaR-immunoadsorbents, and the elution of preparatio containing "high" FcgammaR binders antibodies.

19. A method according to claim 17 to enrich for antibodies that poorly bind FcgammaRI and/or FcgammaRII or that do not bind FcgammaRIII and/or FcgammaRII, wherein sa method comprises the collection of the effluent fraction of said Fcgammal immunoadsorbents, allowing the obtention of preparations containing "low" Fcgamma binders antibodies.

20. A composition comprising at least 80%, preferably at least 95%, of antibodies obtainab by the method according to one of claims 1 to 20.

21. A composition comprising at least 80%, preferably at least 95% of antibodies or peptid displaying at least one (for example 2) of the following features:

a) a MFI higher than 500, more preferably 700, and a percentage of labeled cells higher tha 90 %, more preferably 95 %, in an immunofluorescence assay that measures the binding 5, 10, 25 micrograms/ml antibody (containing less than 2% aggregates as determined b HPLC) to $5x10^5$ CD16 (FcgammaRIII) positive indicator cells (characterized by a number FcgammaRIII receptors lower than 10^5/cell); An example of this is the R297 antibod Another example is the 4B12 antibody.

b) a MFI lower than 80, more preferably 20, and a percentage of labeled cells lower than 5 % , more preferably 2 %, in an immunofluorescence assay that measures the binding of 5, 10, 25 micrograms/ml antibody (containing less than 2% aggregates as determined by HPLC) to $5x10^5$ CD16 (FcgammaRIII) positive indicator cells (characterized by a number of FcgammaRIII receptors lower than 10^5/cell); An example of this is the AD1 antibody. Another example is the B11 antibody.

c) a MFI higher than 50, more preferably 100, and a percentage of labeled cells higher than 80 %, more preferably 90 % in an immunofluorescence assay that measures the binding of 10, 25, 50 micrograms/ml antibody (containing less than 2% aggregates as determined by HPLC) to $5x10^5$ CD32 (FcgammaRIIB) positive indicator cells (characterized by a number of FcgammaRIIB receptors lower than 5×10^4/cell). An example of this is the R297 antibody. Another example is the 4B12 antibody.

d) a MFI lower than 50, more preferably 20, and a percentage of labeled cells lower than 5 %, more preferably 2 %, in an immunofluorescence assay that measures the binding of 10, 25, 50 micrograms/ml antibody (containing less than 2% aggregates as determined by HPLC) to $5x10^5$ CD32 (FcgammaRIIB) positive indicator cells (characterized by a number of FcgammaRIIB receptors lower than 5×10^4/cell). An example of this is the AD1 antibody. Another example is the B11 antibody.

22. A composition comprising at least 80%, preferably at least 95% of antibodies or peptides displaying the feature as defined in c) in claim 21.

23. A composition comprising at least 80%, preferably at least 95% of antibodies or peptides displaying the features as defined in b) and c) in claim 21.

24. A composition comprising at least 80%, preferably at least 95% of antibodies or peptides displaying the features as defined in a) and c) in claim 21.

25. A composition comprising at least 80%, preferably at least 95% of antibodies or peptide displaying the feature as defined in d) in claim 21.

26. A composition comprising at least 80%, preferably at least 95% of antibodies or peptide displaying the features as defined in b) and d) in claim 21.

27. A composition comprising at least 80%, preferably at least 95% of antibodies or peptide displaying the features as defined in a) and d) in claim 21.

28. Use of a composition according to one of claims 22 to 24 to manufacture a medicamen for treating auto-immune diseases or other antibody-related pathologies such as allo immunization.

29. Use of a composition according to one of claims 22 to 24 to manufacture a medicamen for treating allergies and IgE-dependent Type I hypersensitivity.

30. Use of a composition according to one of claims 22 to 24 to manufacture a medicamen for treating allergies.

31. Use according to one of claims 22 to 24 for treating asthma, allergic rhinitis, allergi sinusitis, anaphylactic syndrome, urticaria, angioedema, atopic dermatitis, allergic contac dermatitis and erythema.

32. Use of a composition according to one of claims 25 to 27 to manufacture a medicamen for treating cancer.

33. Use of a composition according to one of claims 22 to 24 to manufacture a medicamen for the regulation of hematopoietic cell proliferation dependent on Receptor Tyrosin Kinases (RTKs) by FcgammaRIIB.

34. Use according to claim 33, wherein said antibodies are bifunctional molecules such as anti-RTK IgG antibodies with optimized Fc region capable of efficiently co-aggregating RTKs with FcgammaRIIB, to manufacture a medicament for blocking the proliferation of FcgammaRIIB+ tumor cells.

35. Use of a composition according to one of claims 20 to 27 to manufacture a medicament, wherein the antibody recognizes antigens expressed on or bound to the cell surface of target cells.

36. Use according to claim 35, wherein said antigens include allo-antigens, transplantation antigens, self-antigens such as class I and class II Major Histocompatibility Antigens (HLA), FcgammaRI, FcgammaRIIA, FcgammaRIII molecules, FcalphaR, FcepsilonRI, B cell receptor, T cell receptor, tumor antigens such as CD20, Her2/NEU, CEA, GD2, allergen such as phospholipase A2, and IgE.

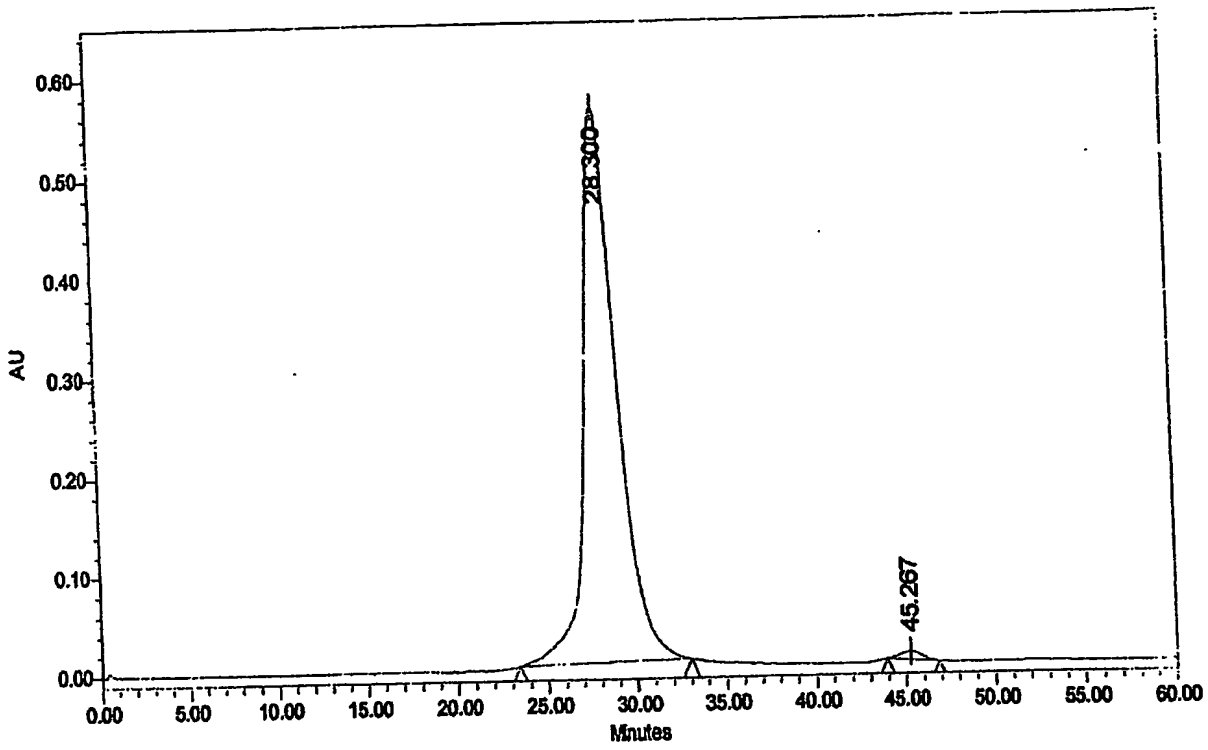
37. Use of a composition according to one of claims 20 to 27, wherein the antibodies recognize Rhesus D antigen to manufacture a medicament for treating or preventing Rhesus alloimmunization of Rh negative patients, leading to the Hemolytic Disease of the New Born (HDNB).

38. Use of a composition according to one of claims 20 to 27, wherein the antibodies recognize Rhesus D antigen to manufacture a medicament for treating or preventing Idiopathic Thrombocytopenic Purpura (ITP) .

39. Use of a composition according to one of claims 20 to 27, wherein the antibodies HLA Class II molecules to manufacture a medicament for treating or preventing cancers, auto-immune diseases, or graft rejections.

ABSTRACT

The present invention relates to a method for the production and the selection of human or chimæric or humanized antibodies or molecules that comprise the Fc region of human IgG, capable of modulating the activity of one or several particular Fc receptors, such as the triggering of inhibitory functions through the human type II receptors of IgG (FcgammaRII/CD32).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0.50
0.40
0.30
0.20
0.10
0.00
AU
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5.00
10.00
15.00
20.00
25.00
30.00
35.00
40.00
45.00
50.00
55.00
60.00
Minutes

FIGURE 1

Oligosaccharide composition of different monoclonal antibodies determined by HPCE-LIF

	Anti-D		Anti-HLA-DR	
Structures	**R297, YB2/0**	**AD1, Heterohybrid**	**4B12, YB2/0**	**B11, CHO**
Fucosylated	**22**	**67**		
Sialylated	**0**	**30**		
A2F	**ND**	**8,7**		
A2FB	**ND**	**ND**		
A1F	**ND**	**21,3**		
A1FB	**ND**	**ND**		
G2FB	**ND**	**ND**		
G2F	**5,7**	**16,8**		
G2	**8,3**	**2**		
G1F	**20,4**	**18,2**		
G1	**35,7**	**1,7**		
G0F	**4,8**	**1,7**		
G0	**27,8**	**0,5**		

ND: not detected

Different forms of gycans :

A: sialilated

G0 : agalactosylated

G1 : monogalactosylated

G2 : bigalactosylated

F : fucosylated

B : bisecting GlNAc

FIGURE 2

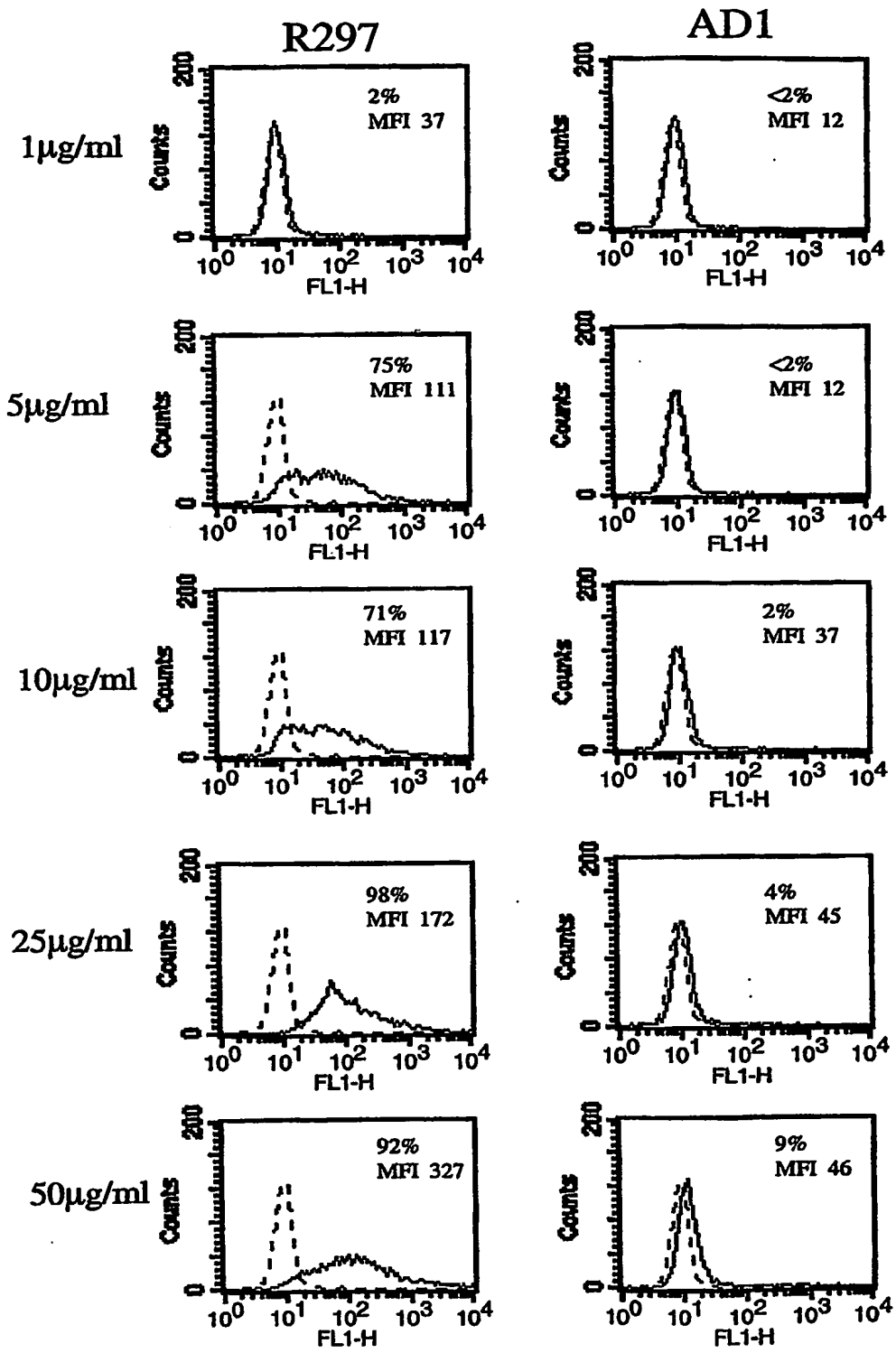

R297
AD1
1µg/ml
5µg/ml
10µg/ml
25µg/ml
50µg/ml
2%
MFI 37
<2%
MFI 12
75%
MFI 111
<2%
MFI 12
71%
MFI 117
2%
MFI 37
98%
MFI 172
4%
MFI 45
92%
MFI 327
9%
MFI 46
Counts
FL1-H

FIGURE 3

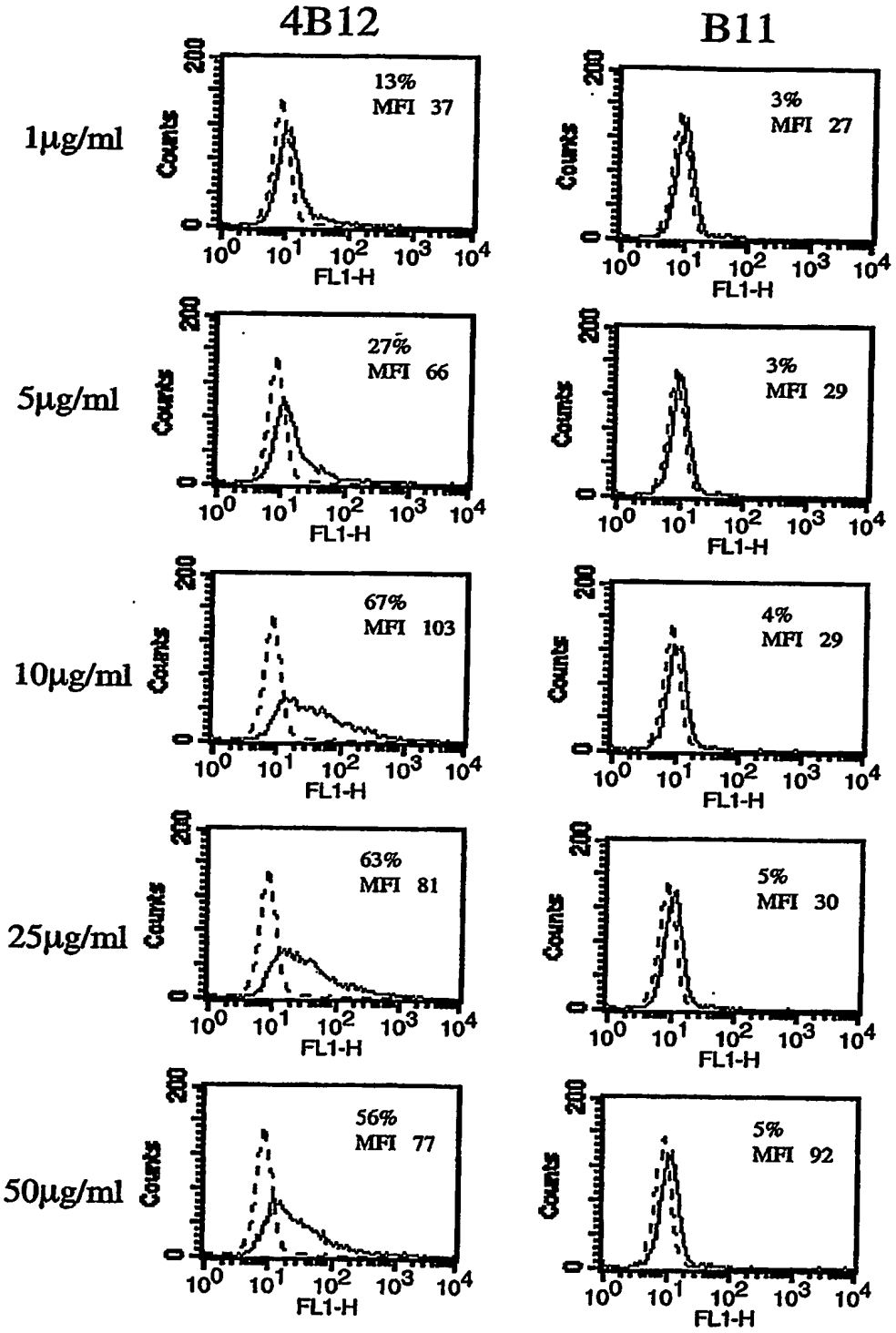

4B12
B11
1µg/ml
5µg/ml
10µg/ml
25µg/ml
50µg/ml
13% MFI 37
3% MFI 27
27% MFI 66
3% MFI 29
67% MFI 103
4% MFI 29
63% MFI 81
5% MFI 30
56% MFI 77
5% MFI 92
Counts
FL1-H

FIGURE 4

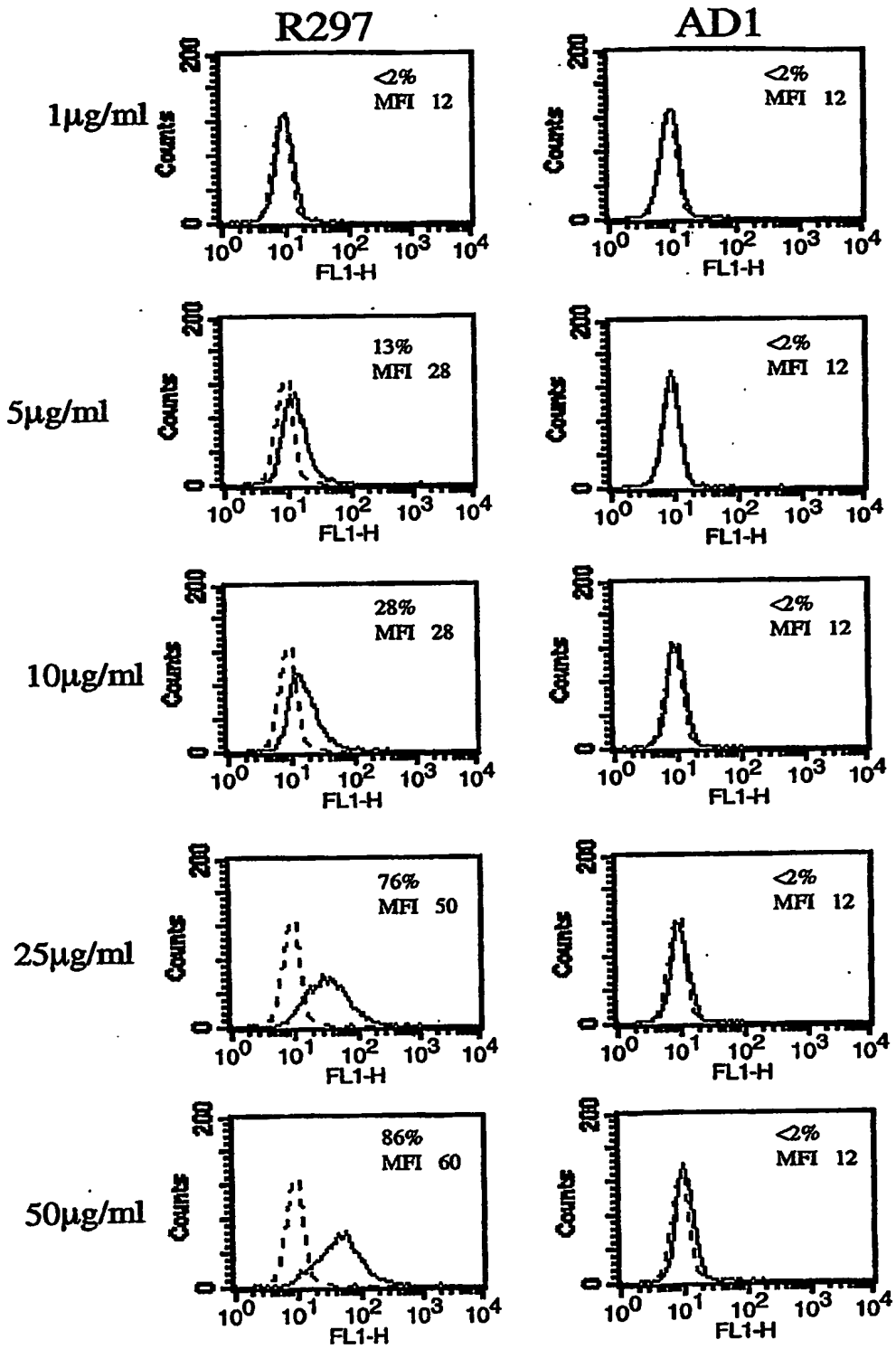

R297
AD1
1µg/ml
5µg/ml
10µg/ml
25µg/ml
50µg/ml
<2%
MFI 12
13%
MFI 28
28%
MFI 28
76%
MFI 50
86%
MFI 60
Counts
FL1-H

FIGURE 5

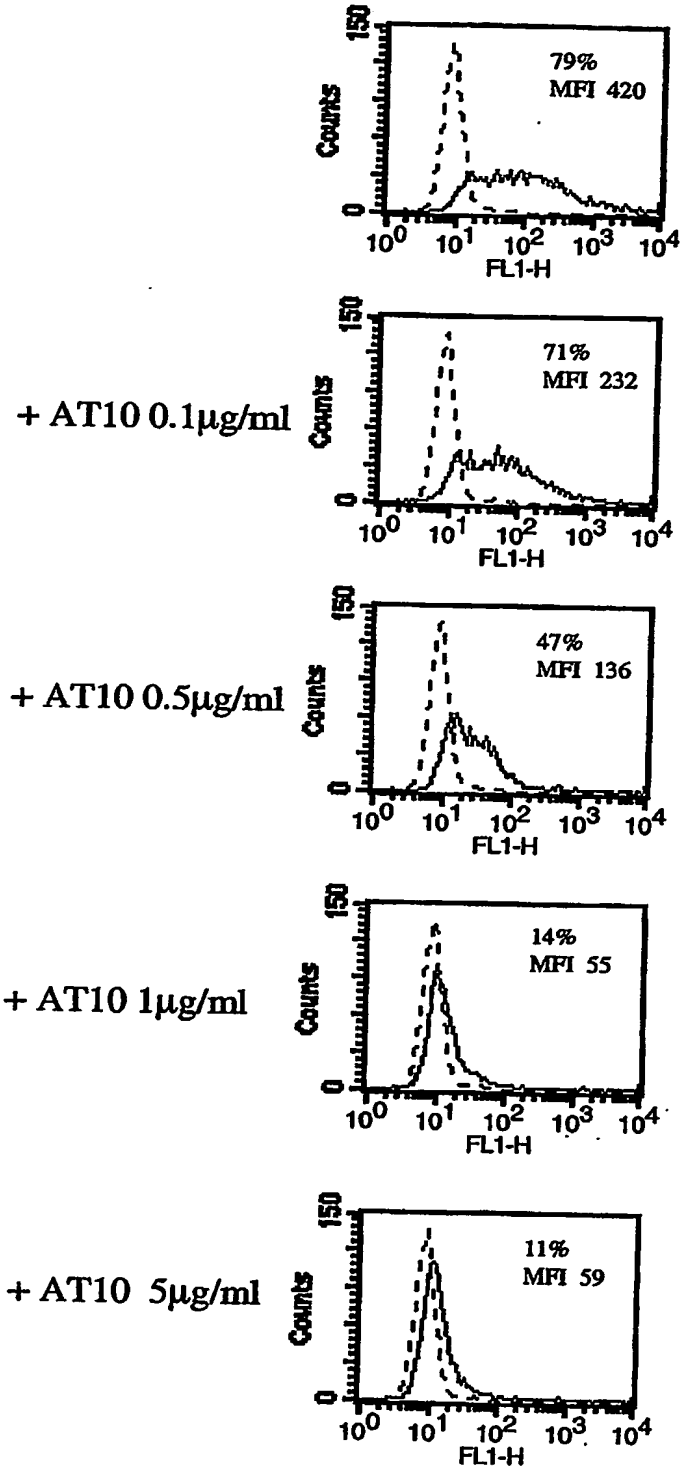
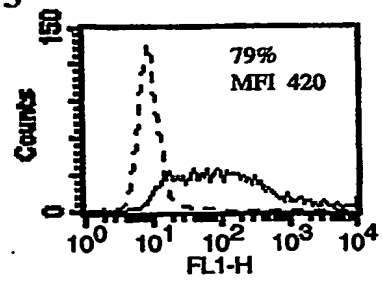
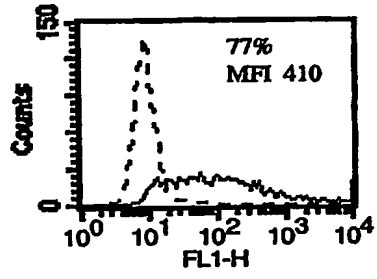

79%
MFI 420
+ AT10 0.1µg/ml
71%
MFI 232
+ AT10 0.5µg/ml
47%
MFI 136
+ AT10 1µg/ml
14%
MFI 55
+ AT10 5µg/ml
11%
MFI 59
Counts
150
0
10^0 10^1 10^2 10^3 10^4
FL1-H

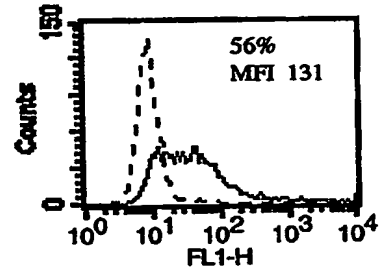
FIGURE 6



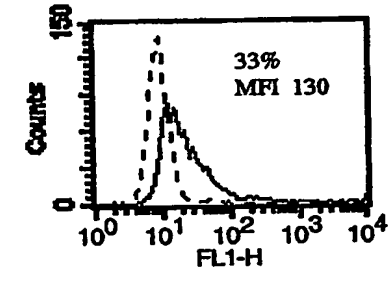
+ 6C4 1µg/ml



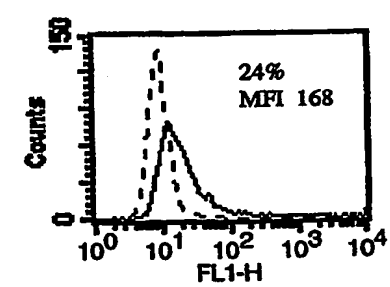
+ 6C4 10µg/ml



+ 6C4 25µg/ml



+ 6C4 50µg/ml



+ 6C4 100µg/ml

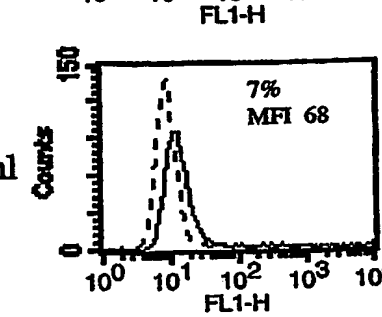


FIGURE 7

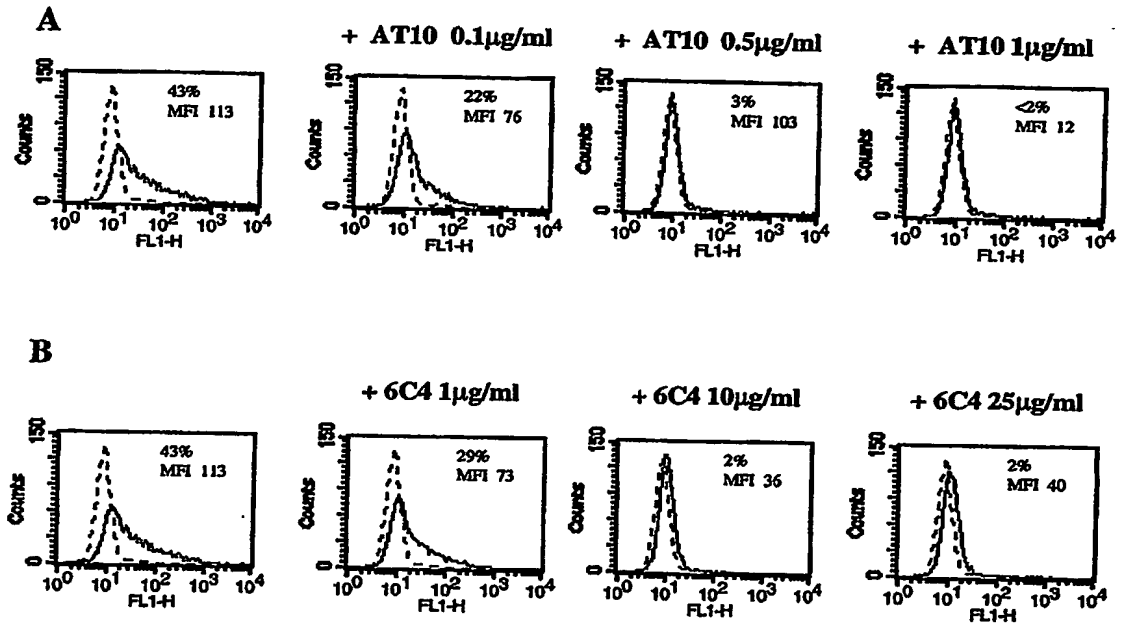
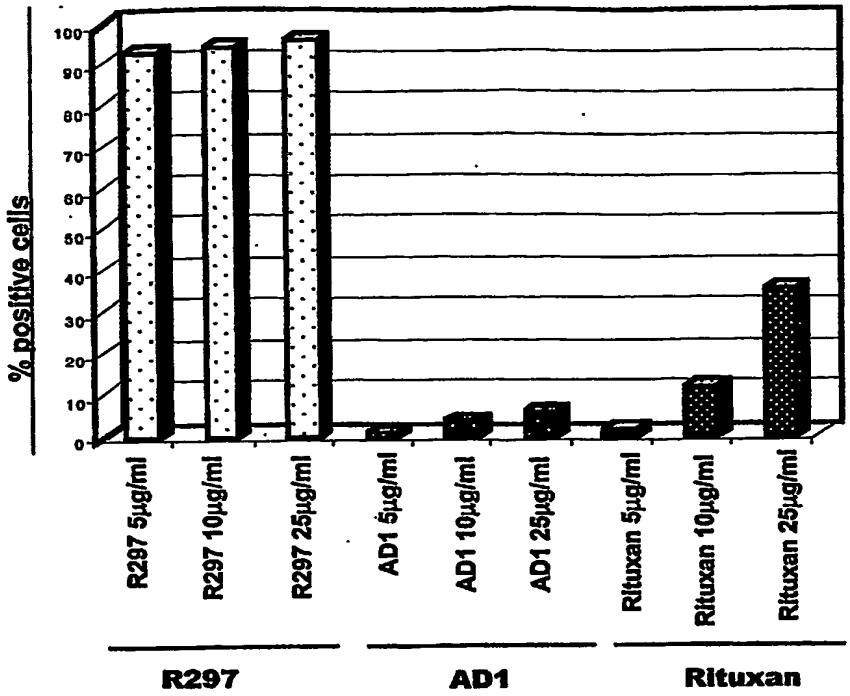

A
+ AT10 0.1µg/ml
+ AT10 0.5µg/ml
+ AT10 1µg/ml
43%
MFI 113
22%
MFI 76
3%
MFI 103
<2%
MFI 12
B
+ 6C4 1µg/ml
+ 6C4 10µg/ml
+ 6C4 25µg/ml
43%
MFI 113
29%
MFI 73
2%
MFI 36
2%
MFI 40
Counts
FL1-H

FIGURE 8


% positive cells
100
90
80
70
60
50
40
30
20
10
0
R297 5µg/ml
R297 10µg/ml
R297 25µg/ml
AD1 5µg/ml
AD1 10µg/ml
AD1 25µg/ml
Rituxan 5µg/ml
Rituxan 10µg/ml
Rituxan 25µg/ml
R297
AD1
Rituxan

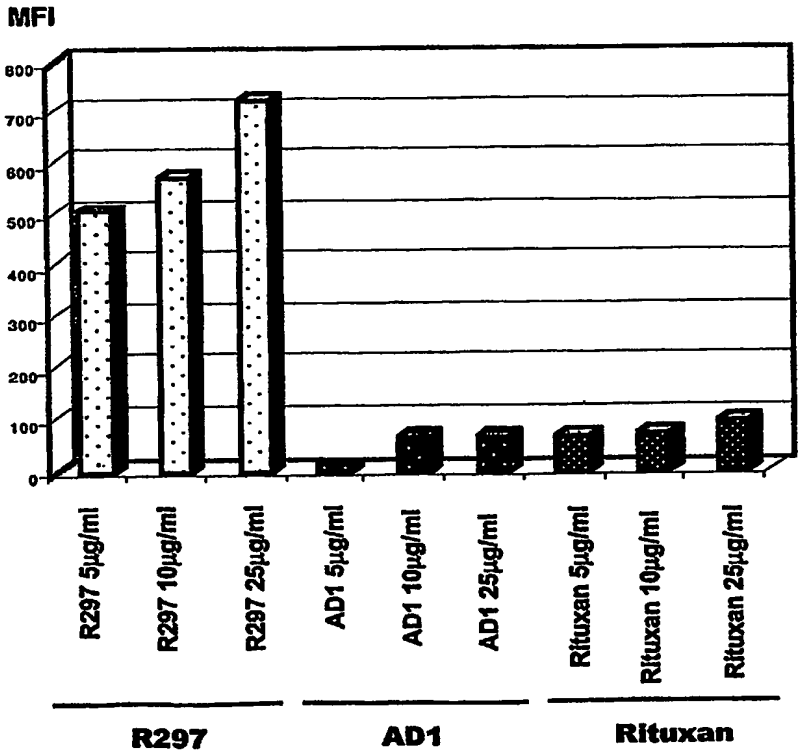
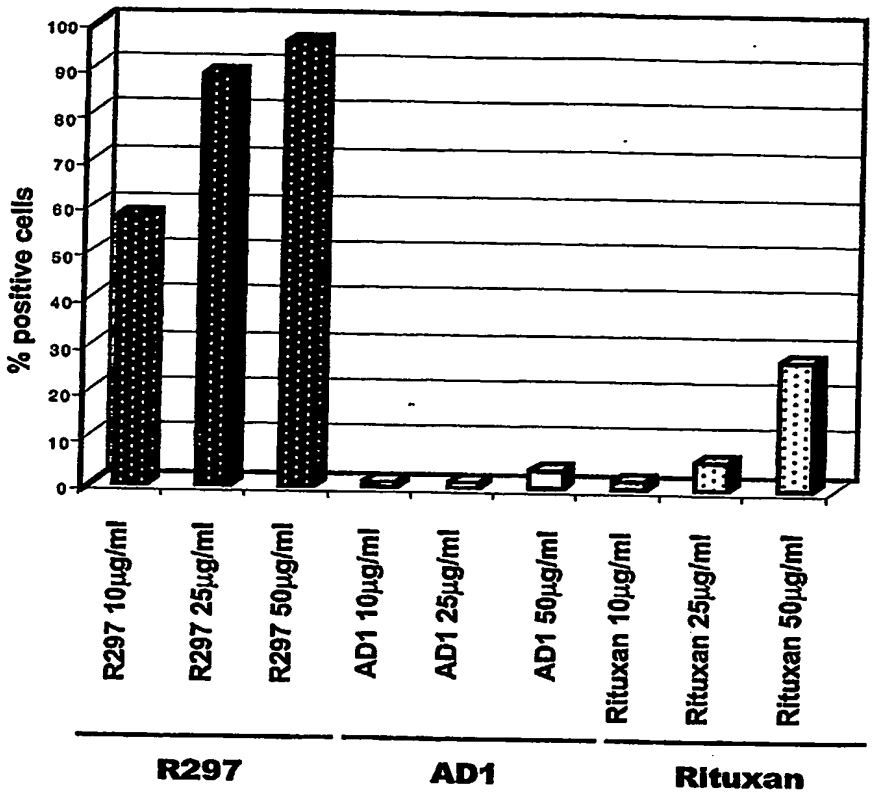

MFI
800
700
600
500
400
300
200
100
0
R297 5µg/ml
R297 10µg/ml
R297 25µg/ml
AD1 5µg/ml
AD1 10µg/ml
AD1 25µg/ml
Rituxan 5µg/ml
Rituxan 10µg/ml
Rituxan 25µg/ml
R297
AD1
Rituxan

FIGURE 9


% positive cells
100
90
80
70
60
50
40
30
20
10
0
R297 10µg/ml
R297 25µg/ml
R297 50µg/ml
AD1 10µg/ml
AD1 25µg/ml
AD1 50µg/ml
Rituxan 10µg/ml
Rituxan 25µg/ml
Rituxan 50µg/ml
R297
AD1
Rituxan

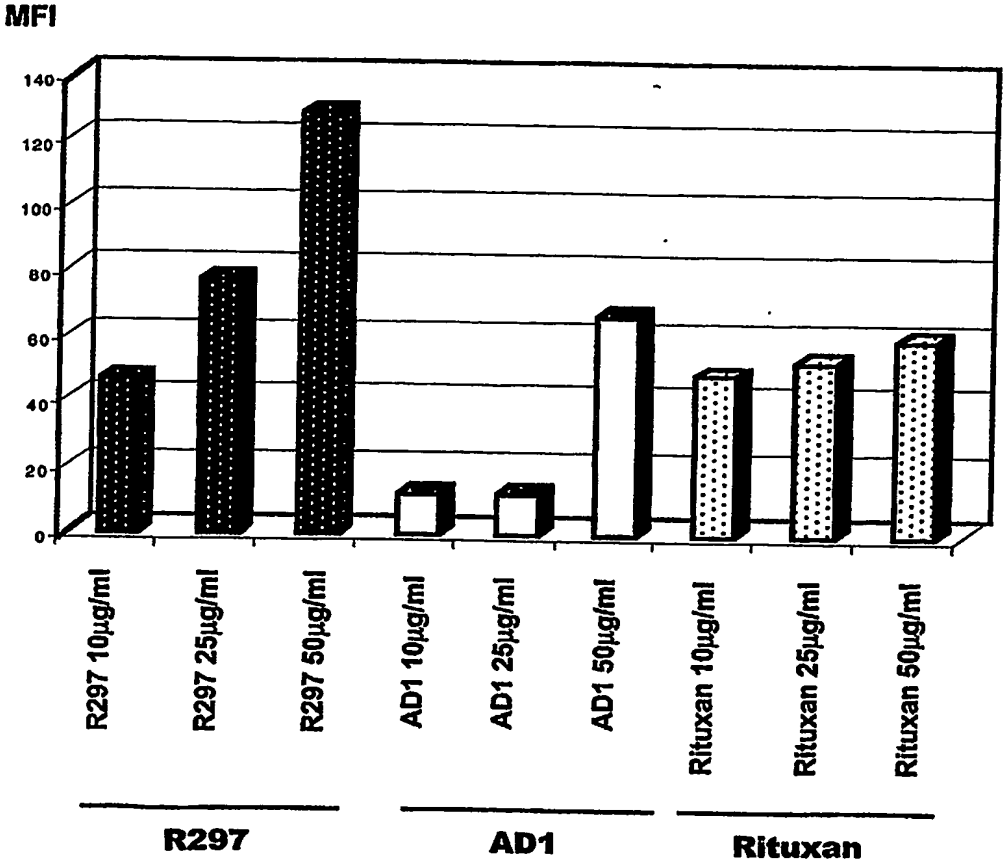

MFI
140
120
100
80
60
40
20
0
R297 10µg/ml
R297 25µg/ml
R297 50µg/ml
AD1 10µg/ml
AD1 25µg/ml
AD1 50µg/ml
Rituxan 10µg/ml
Rituxan 25µg/ml
Rituxan 50µg/ml
R297
AD1
Rituxan

FIGURE 10

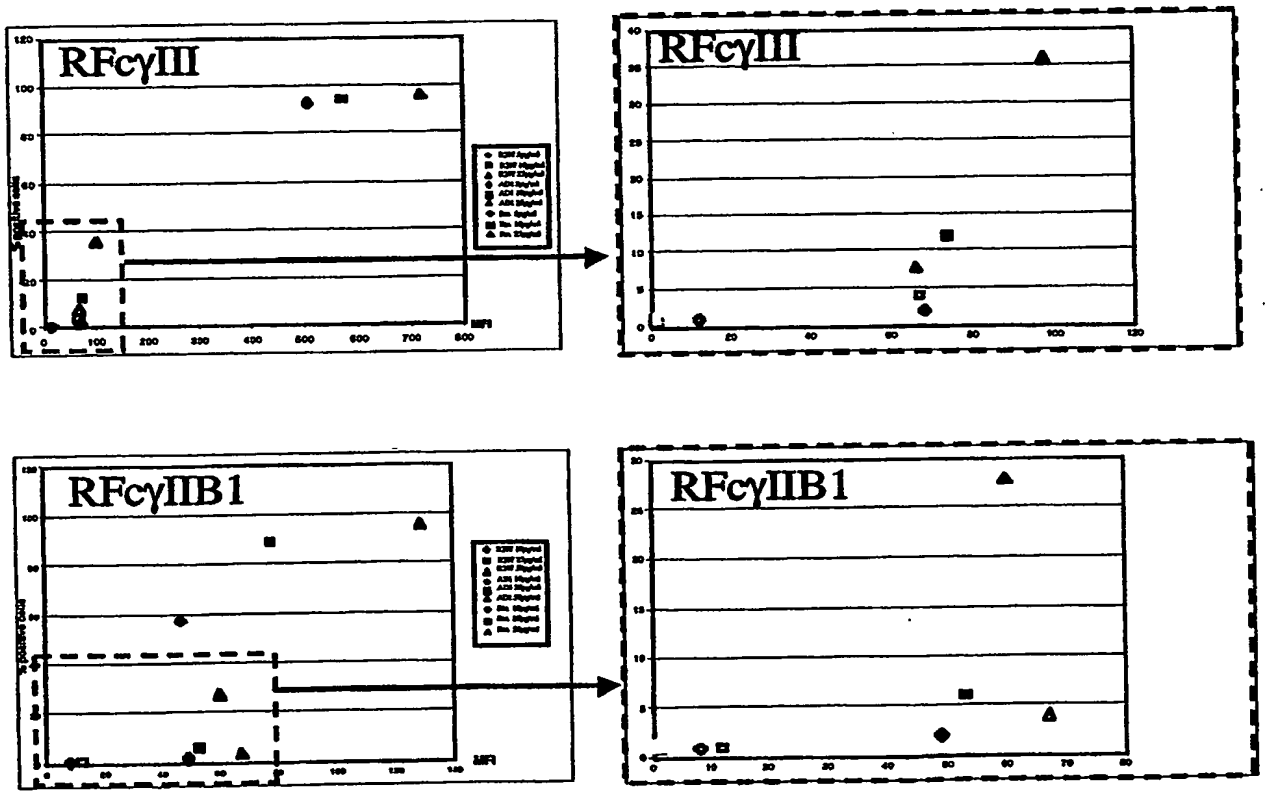


FIGURE 11

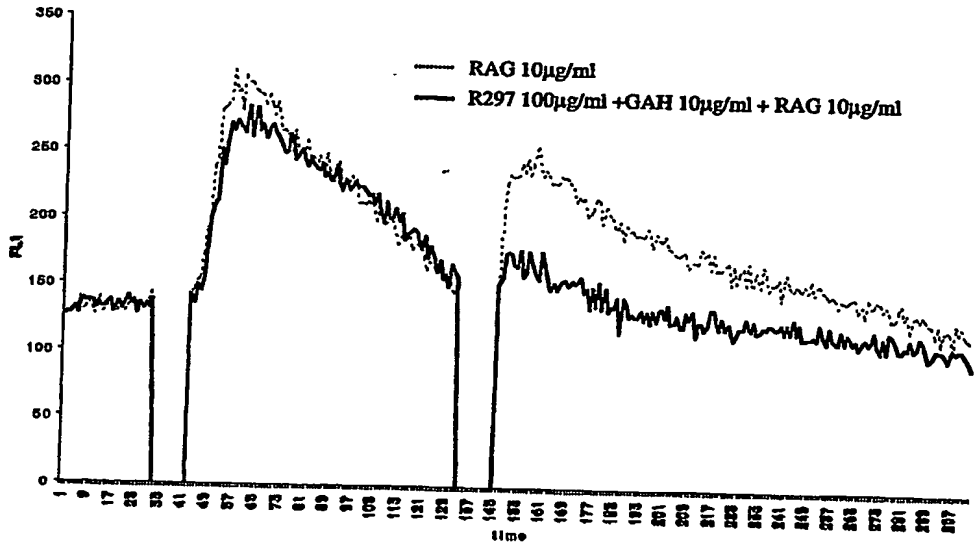


FIGURE 12

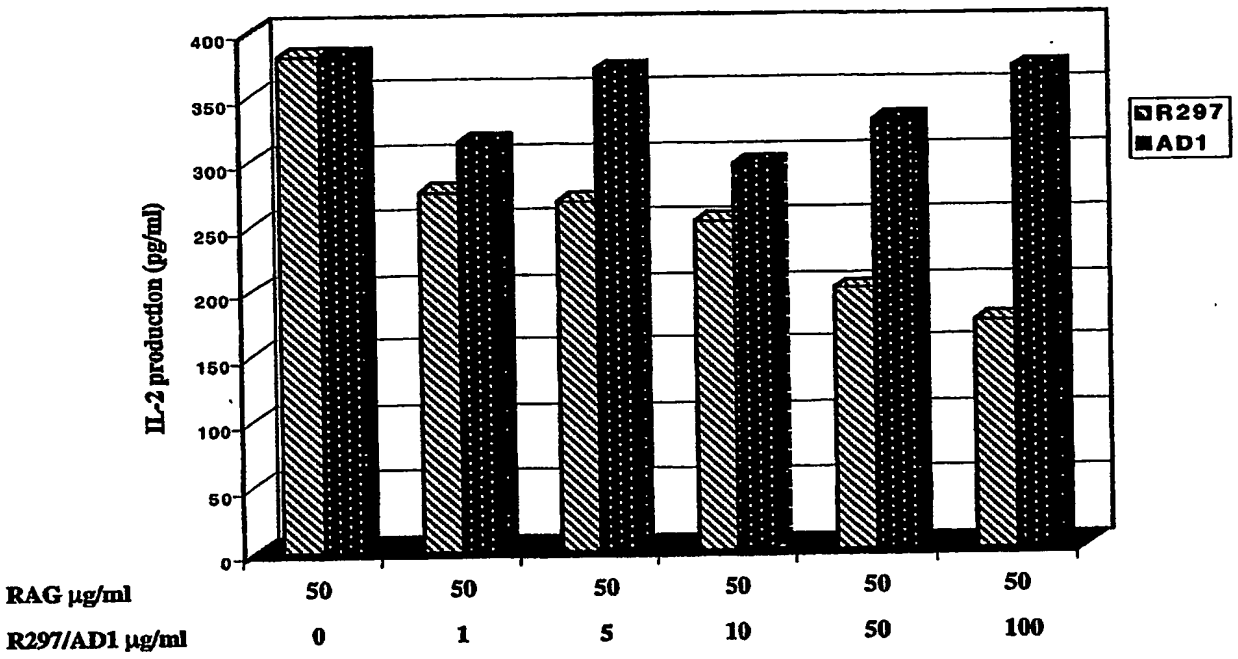

IL-2 production (pg/ml)
400
350
300
250
200
150
100
50
0
R297
AD1
RAG µg/ml
R297/AD1 µg/ml
50 50 50 50 50 50
0 1 5 10 50 100

FIGURE 13

SEQUENCE LISTING

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<110> INSTITUT NATIONAL DE LA SANTE ET DE LA RECHERCHE MEDICALE (INSERM)

<120> THERAPEUTIC PRODUCTS WITH ENHANCED ABILITY TO IMMUNOMODULATE CELL FUNCTIONS

<130> D21018

<160> 4

<170> PatentIn version 3.2

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<220>

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atcggatccc gactgaagat ctc 23